

Maternal High Fat Feeding:  
Impact of Female Offspring Body Composition and Bone Health

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## ABSTRACT

High fat diet (HFD) consumption in rodents alters body composition and weakens bones. Whether female offspring of mothers consuming a HFD are similarly affected at weaning and early adulthood is unclear. This research determined whether maternal HFD contributes to long-lasting alterations in body composition and bone health of female offspring. Rats were fed control or HFD for 10 weeks prior to and throughout pregnancy and lactation. Female offspring were studied at weaning or 3 months of age (consumed control diet). Main findings in female offspring: maternal HFD decreased lean mass, increased fat mass and femoral BMD at weaning, but not at 3 months; weanling femoral lipid composition reflected maternal diet, persisting to 3 months of age (decreased total and *n*6 polyunsaturates, increased saturates); and no differences in femoral strength at 3 months. In summary, 3 month old female offspring have similar body composition and bone health regardless of maternal diet.

## KEY WORDS

Bone, body composition, female offspring, high fat diet, nutritional programming

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## LIST OF ABBREVIATIONS

BMC – bone mineral content

BMD – bone mineral density

CT – computed tomography

CON – control

DNA – deoxyribonucleic acid

DXA – dual-energy x-ray  
absorptiometry

ER $\alpha$  – estrogen receptor alpha

HRT – hormone replacement therapy

HSF – high saturated fat

IL-6 – interleukin-6

LR – leptin receptor

LV – lumbar vertebra(e)

MSC – mesenchymal stem cell

$\mu$ CT – micro-computed tomography

MCP-1 – monocyte chemoattractant  
protein-1

MUFA – monounsaturated fatty  
acids/monounsaturates

OPG – osteoprotegrin

PTH – parathyroid hormone

PPAR – peroxisome proliferator-  
activated receptor

PUFA – polyunsaturated fatty  
acids/polyunsaturates

PND – postnatal day

RANK – receptor activator of nuclear  
factor kappa-B

RANKL – receptor activator of nuclear  
factor kappa-B ligand

ROI – region of interest

SFA – saturated fatty acids/saturates

TNF- $\alpha$  – tumour necrosis factor-alpha

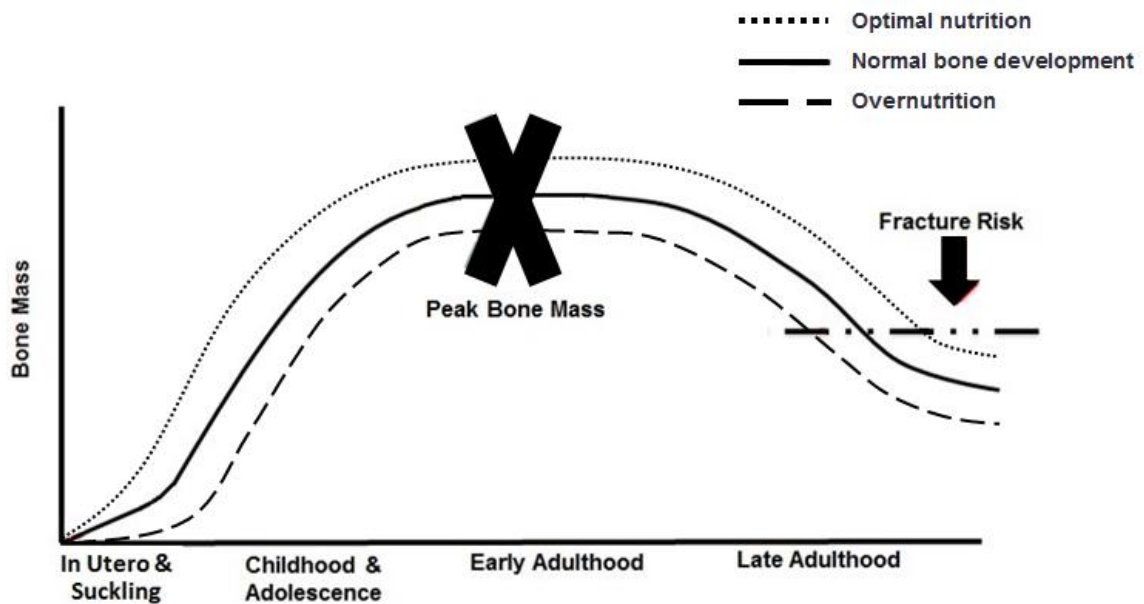
## **Chapter 1.0: Introduction**

### **1.1. Osteoporosis in Canada**

Osteoporosis is a disease in which bones have become weakened through gradual loss of mineral and structural integrity, leaving bones fragile and more susceptible to fragility fracture. The incidence of osteoporotic fracture in Canada is among the highest in the world, ranked in the top quarter internationally [1]. According to the 2009 Canadian Community Health Survey conducted by the Public Health Agency of Canada, the incidence of osteoporosis was reported at 1.5 million or 10% of Canadian adults 40 years or older [2]. Osteoporosis Canada reported that the incidence of osteoporotic fractures is greater than the incidence of breast cancer, heart attack and stroke combined [1]. Once an individual has suffered from an osteoporotic fracture they are at an increased risk of additional fractures. The prognosis for recovery from major osteoporotic fractures is grave; with hip and vertebral fractures being the most severe. Approximately 28% of Canadian women and 37% of men who suffer from hip or vertebral fractures will die within a year [1]. It is therefore of utmost importance for Canadians to develop prevention strategies that improve quality of life by reducing the incidence of fragility fractures, as well as mortality due to osteoporosis.

Some purport that osteoporosis is a ‘a pediatric disease with geriatric consequences’ [3]. The quantity of bone acquired by late adulthood is dependent on the amount of bone gained during childhood and adolescence. Optimal bone mass in adolescence can be achieved if an individual achieves their peak bone mass, which is the genetically predisposed limit of bone mass one can possibly attain [4]. The amount of bone accrued during childhood and adolescence will determine the amount of bone mass

one has in adulthood; where individuals who have reached peak bone mass are likely to have higher bone mass in adulthood [5]. Therefore, if peak bone mass is not achieved, there is a greater risk of developing osteoporosis-related fractures in the future, as bone loss with aging is inevitable. For instance, it has been shown that only a 10% increase in peak bone mass can reduce the incidence of hip fractures by thirty percent [6]. The attainment of peak bone mass occurs in early adulthood, which is depicted in Figure 1. Genetics contributes to approximately 60-80% of peak bone mass, however other lifestyle factors such as nutrition can alter the predisposed limit [7]. Figure 1 displays a



**Figure 1.** Schematic of the role of nutrition on peak bone mass and fracture risk

schematic of the role nutrition plays on peak bone mass. Overnutrition (e.g. high energy intake, inadequate nutrient consumption), represented by long hashed lines, can lower peak bone mass, and subsequently increase the risk of fragility fracture earlier in life. Optimal nutrition with adequate intake of important nutrients that support bone health

(e.g. calcium and vitamin D), represented by short hashed lines, may lead to a higher peak bone mass, and thereby delay the risk of fracture until later in life. It is therefore of considerable importance to understand how certain modifiable lifestyle factors such as diet can help improve peak bone mass development and promote overall bone health. Furthermore, whether specific nutrients can influence peak bone mass should be investigated further, especially considering the high incidence of osteoporosis and increased mortality associated with fragility fractures.

## **1.2. Bone development and growth**

### **1.2.1. Bone development**

Bone development begins in utero by two processes known as intramembranous and endochondral ossification [8, 9]. Intramembranous ossification is responsible for formation of the skull and facial bones [10]. It begins with the formation of limb buds, an accumulation of mesenchymal stem cells, which is controlled by many genes, particularly the Hox family genes [11]. Mesenchymal stem cells become condensed and highly vascularized [9], and differentiate to osteoprogenitor cells by transcription factor Runx2 [11, 12]. Eventually these osteoprogenitor cells further differentiate to osteoblasts by osterix, and express Runx2 and type I collagen. These osteoblasts can then excrete osteoid matrix that later becomes mineralized [12]. Osteoblasts either succumb to apoptosis or become mature osteocytes that remain embedded in the bone matrix [12]. The mineralized osteoid matrix forms bone spicules that later help form the lamellae of the Haversian canals [9].

Endochondral ossification is responsible for bone formation in the majority of the skeleton [10], particularly long bones, while vertebral and pelvic bone formation is a product of the combination of intramembranous and endochondral ossification [11]. The main difference between intramembranous and endochondral ossification is the fate of mesenchymal stem cells. In intramembranous ossification, mesenchymal stem cells differentiate directly to bone, whereas mesenchymal stem cells in endochondral ossification differentiate to cartilage, which acts as a template for future bone growth [12]. Long bone formation is initiated with the connection and condensation of mesenchymal stem cells that subsequently become loose mesenchyme, coordinated by the transcription factor Sox9 [11]. This forms the limb bud or initial template for further bone formation [12]. Sox9 also initiates the differentiation of condensed mesenchymal stem cells to chondrocytes [10, 11], that begin to lay down a cartilage matrix composed of type II collagen [12, 13]. Chondrocytes further mature and proliferate to hypertrophic cartilage, exiting the cell cycle, and secrete extracellular matrix that expresses collagen X [13] instead of type II collagen in the cartilage matrix [12]. Hypertrophic chondrocytes secrete factors to promote angiogenesis, which subsequently promotes transportation of osteoblasts and osteoclasts, as well as hematopoietic cells to cartilage [13]. The addition of these cells helps create primary ossification centres to allow for bone formation to occur. At this site, the hypertrophic chondrocytes undergo apoptosis, and osteoblasts help to build bone after increasing accumulation of calcium and enzymes such as metalloproteinase and alkaline phosphatase to promote mineralization [11].

The primary ossification centres of long bones begin in the diaphysis, and with the proliferation of chondrocytes, the epiphyses begin to form at the end of long bones.

These chondrocytes undergo hypertrophy and eventually undergo apoptosis, which provides a template for new trabecular bone to be formed [12]. The periosteum begins to form at the periphery through intramembranous ossification from the middle of the diaphysis towards the epiphysis [14]. The periosteum is composed of an outer layer of chondrocytes and an inner layer of osteoprogenitor cells that eventually differentiate to osteoblasts, and finally mature osteocytes [12, 14]. At the newly formed epiphyses, cartilage canals develop with vascularization. This allows for a mass of chondrocytes that begin to hypertrophy at the centre of the epiphysis, which will become the epiphyseal growth plate. This occurrence also initiates the formation of the secondary ossification centre [14].

Bone formation occurring at the secondary ossification centre occurs postnatally, after the primary ossification centres have been formed in utero. New vascularization occurs, which brings preosteoblasts and mesenchymal stem cells to the ossification centre. The previously formed matrix that is proximal to the hypertrophied chondrocytes also becomes mineralized at this time. The previously formed cartilage in the epiphysis becomes replaced by hematopoietic cells and the secondary ossification centre as it continues to grow [14]. The remaining cartilage at the epiphysis goes through its final stages of ossification and becomes bone, also known as the growth plate. At this time, fat is present in the secondary ossification centre. Blood vessels from the marrow of the secondary ossification centre begin to spread to the growth plate and physis (proximal to growth plate). Finally, in the last stages of endochondral ossification, the hematopoietic marrow turns into fat [14].

Bone formation and mineral accretion (calcium, phosphorus, magnesium) is at its greatest in the third trimester, however these processes remain highly active in early infancy as well. This rate remains high for the first months of infancy, but slows with age [15]. Mineral accretion due to delivery from mother's milk is important in infancy as it supports optimal bone and somatic growth. Bone formation remains rapid during childhood and adolescence, particularly long bone appositional growth. This growth occurs at a fast rate until peak bone mass is attained. At this point in life (early adulthood), bone formation is considerably slower, and bone remodelling takes over [16]. Bone is continuously remodelled through the coupling of bone formation and bone resorption throughout adulthood, which will be discussed further in section 1.2.2.

Maternal nutrition may also augment offspring bone development, as offspring are still being nourished from their mothers in critical and rapid periods (in utero and suckling) of bone growth and development. It has been shown that the composition of mother's diet reflects the nutrient composition of the milk [17, 18]. Therefore, maternal diet will determine the composition of nutrients being delivered to offspring, which may have the potential to augment offspring bone development. For instance, female Wistar rats fed a diet restricted in protein had offspring with lower bone mineral density [19]. Furthermore, mothers with insufficient (11-20 µg/L) or deficient (<11 µg/L) levels of vitamin D during late pregnancy were more likely to have children with lower whole body and lumbar spine bone mineral density at 9 years of age [20]. In a society with an abundance of food availability and high prevalence of obesity, it is of concern whether over-nutrition or increased consumption of a particular nutrient (i.e. elevated levels of dietary fat) can augment or impair bone development when exposure occurs in utero and

during suckling. This concept of maternal nutrition and offspring bone health will be explored further in section 3.2.

#### 1.2.2. Bone remodelling

Bone remodelling is a dynamic process in which bone formation and bone resorption are tightly coupled and regulated. The balance between the rate of bone formation and bone resorption govern overall bone homeostasis. For instance, a faster rate of bone formation compared to resorption will favour bone growth. The rates of bone formation and resorption are dictated primarily by the action and number of osteoblasts and osteoclasts [21], which originate from bone marrow. Osteoblasts are mononuclear cells derived from mesenchymal stem cells, as mentioned in section 1.2.1. Mesenchymal stem cells (MSCs) become preosteoblasts through the presence of Runx2 or Cbfa1 [22, 23], and again differentiate to functional osteoblasts through osterix [23]. Adipocytes are also differentiated from these pluripotent MSCs, and the presence or absence of certain transcription factors (e.g. PPAR $\gamma$ ) may shift differentiation of the MSC to favour osteogenesis or adipogenesis during development [24]. The primary role of osteoblasts is to regulate bone formation, both by depositing matrix that becomes mineralized and by limiting resorption. Osteoblasts secrete bone matrix, more specifically collagenous and non-collagenous proteins. Typically, bone is composed of ~90% type I collagen, which is important structurally as it provides the template for further bone deposition and formation [25].

Unlike osteoblasts, osteoclasts are multinucleated macrophage-type cells that differentiate from hematopoietic stem cells [26, 27]. Growth factor colony stimulating



factor, or macrophage colony-stimulating factor secreted by osteoblasts, turns the hematopoietic stem cells to preosteoclasts. Preosteoclasts then associate directly with the osteoblast cell through its receptor activator of nuclear factor-  $\kappa$ B (RANK), and attaches itself to the RANK-ligand (RANKL) presented on the osteoblast membrane. This binding promotes osteoclastogenesis and furthermore osteoclast apoptosis [28]. Osteoclasts become activated through binding of RANKL to RANK, which allows osteoclasts to proliferate, develop a ruffled border, and polarize. Once polarized, osteoclasts are able to attach to the lining of old bone, and release acids and proteases (e.g. matrix metalloproteinase 9 [29]) that breakdown bone [30].

The rate of osteoclastogenesis is regulated by osteoblasts, through the production of a decoy receptor osteoprotegrin (OPG) [27, 31]. RANKL will bind to OPG, thereby preventing binding to RANK and subsequent differentiation of preosteoclasts, lowering the rate of osteoclastogenesis and subsequently bone resorption. Furthermore, the number of OPG present can vary according to the presence or absence of certain factors. For example, the addition of estrogen to human osteoblasts *in vitro* causes increased gene and protein expression of OPG [32]. Therefore greater expression of OPG increases the likelihood of RANKL to bind, subsequently reducing osteoclast maturation and resorption.

The process of bone remodelling occurs constantly over the lifespan, and can vary according to age. In childhood and adolescence, a period of rapid growth, bone remodelling will favour bone formation rather than resorption, resulting in bone growth and acquisition. However at early adulthood, once peak bone mass has been attained, bone formation and bone resorption become coupled, resulting in a net loss and gain of

bone. Aging causes bone remodelling to favour bone resorption over formation, resulting in bone loss that can lead to the development of osteoporosis.

### **1.3. Diet, obesity, and inflammation in Canada**

The typical Canadian diet, or Western diet, is comprised of higher than recommended intakes of fat, specifically saturated fat [33], and refined sugars. There is no particular recommendation on the level of saturated fat one should obtain in their diet, as high saturated fat intake is associated with the development of cardiovascular disease[34]. However, Health Canada recommends consuming minimal amounts of saturated fat in a nutritious balanced diet. The Canada Food Guide outlines the suggested servings per day of food groups that contain essential nutrients to support a healthy lifestyle (i.e. fruits and vegetables, meat and alternatives). By following the Canada Food Guide, an individual would consume only 5.8-7.2% of their daily energy intake from saturated fat [34]. However, based on the Canada Community Health Survey, the mean daily energy intake of saturated fat is ~10% for Canadian adults, which is higher than what one would consume eating a nutritious diet and following the Canada Food Guide. Furthermore in Canada, 23% of females and 25% of males have total dietary fat consumption above the Acceptable Macronutrient Distributable Range [35] (amount of nutrient acceptable for consumption that will not lead to the development of disease [36]). Although regularly consuming a diet high in saturated fat is known to be associated with cardiovascular disease development, the effect on bone health in humans is not as well defined. This novel association may be important to investigate considering the high

mortality rate after an osteoporotic fracture [1], and the increased consumption of dietary fat in Canada.

The role of dietary saturated fat on the achievement of peak bone mass and maintenance of bone health is not well investigated in humans. Some studies have reported increased incidence of fractures in overweight and obese children [37, 38], however it is difficult to determine whether dietary fat, sugar, or the combination of both can be attributed to these observations. Since diet during childhood and adolescence can partly determine peak bone mass [4], it is important to investigate whether higher dietary saturated fat intake during this life stage leads to a predisposition of osteoporosis in adulthood.

Although the relative contribution of dietary saturated fat consumption in childhood to the risk of developing osteoporosis is not well characterized, it is well known that a diet rich in saturated fats can lead to other health complications [33]. A diet high in saturated fat may lead to weight gain and subsequent changes in body composition. Body composition can be monitored by measuring the ratio of lean and fat mass, with a lower ratio being more favourable for mortality [39]. Further monitoring of body fat distribution is an important indicator of risk of disease development and relative morbidity. Individuals with greater fat mass in the abdomen (visceral fat) are at increased risk of developing chronic diseases [40]. Although a diet high in saturated fat may not directly cause abdominal obesity, greater caloric intake along with a sedentary lifestyle can lead to weight gain, when considering the typical Canadian diet [41]. Since adipose tissue is considered an endocrine organ, the levels of circulating hormones released by the adipose tissue can also be altered when the quantity of fat mass is altered. For

example, the increased release of some pro-inflammatory cytokines is associated with chronic inflammation, often observed in obesity [42].

Because evidence from population studies are limited in terms of the direct impact of dietary saturated fats on bone health and body composition and its relative contribution to disease development with high consumption, animal models are useful to elucidate how a diet high in saturated fat modulates bone health and body composition. The role of a high saturated fat diet on body composition, as well as bone health and hormones contributing to overall health in animal models will be discussed further in chapter 2.0.

## **Chapter 2.0: High fat diet consumption**

### **2.1. Effect of high fat diet consumption on rodent body composition**

A diet high in saturated fat can contribute to elevated body weight [43] and greater fat mass in rodents [44], which, when occurring in humans, is associated with a higher risk of mortality [39]. The distribution of fat mass, or where fat accumulation is greatest, is a more accurate predictor of disease development and health status than body weight and fat mass alone. In humans, it is well known that greater fat accumulation in the abdomen increases the risk of developing chronic diseases associated with metabolic syndrome [45], as well as cardiovascular disease [46]. A common tool for determining the degree of abdominal adiposity is by measuring waist circumference in humans [45, 47]. Animal models are commonly used for high fat feeding and obesity studies, with certain well-established models being the most appropriate. Rodent models, such as certain strains of mice (i.e. C57BL/6) and rats (i.e. Wistar, Sprague Dawley) are best suited for this as they are more likely to become obese and develop associated

comorbidities (e.g. insulin resistance) when fed a high fat diet [48]. Studies using these models can provide greater insight into the potential role of dietary fat, fat mass distribution and disease development. Since a diet high in saturated fat may elicit elevations in fat mass, it is likely that abdominal fat mass will also increase, increasing the risk of developing and dying prematurely from a chronic disease.

Another common tool for determining body composition in both humans and animal models is dual-energy x-ray absorptiometry (DXA), which measures whole body, or regional measures of lean and fat mass, as well as bone mineral content. Studies using male and female rodent models have demonstrated that a high fat diet (39% - 59% kcal from fat) can lead to a higher fat mass [49, 50] , and lower lean mass [44]. These studies fed diets that were either high in saturated fat [44, 50], or high in monounsaturated fatty acids and/or polyunsaturated fatty acids [49, 51], each of which elicited changes in body composition (higher fat mass [49, 50] or lower lean mass [44]). Whole body bone mineral content is also impacted by the consumption of a high fat diet. A study in growing male rats has shown that bone mineral content is lowered after 10 weeks of high fat feeding (38.5% kcal from fat – vegetable oil) in the absence of obesity [51]. Investigators of this study speculate that increases in fat mass, particularly visceral fat mass, negatively impacts bone accretion, which may interfere with calcium deposition during bone modeling [51]. Lower whole body bone mineral content or bone mass may lead to weaker bones that are more prone to fracture. From the evidence provided, it is clear that a high fat diet can change body composition, which is of particular concern given the typical Canadian diet is higher in fat intake, and the steady rise in obesity and associated chronic diseases such as cardiovascular disease and type II diabetes [34].

## **2.2. Effect of high fat diet consumption on rodent bone health**

Bone strength, characterized by two important components - bone *quantity* and bone *quality* - is the hallmark for determining overall bone health status in animal models. Bone *quantity* is the amount of bone mineralized, which is described as bone mineral content (BMC) and bone mineral density (BMD). Bone mineral density is determined by calculating the amount of bone deposited (BMC) in a specific area, which is measured using DXA. Bone *quality* is characterized by the structure of cortical and trabecular bone. Bone quality is often examined using specialized imaging equipment such as computed tomography (CT) which can provide details of cortical and trabecular bone structure. The specific components of bone microarchitecture that provide information on overall bone quality in animal models are discussed further in section 2.2.1.

Although bone strength is the hallmark of determining overall bone health and relative fracture risk, it is difficult to directly measure in humans. DXA is unable to measure bone strength, however it provides information on overall bone mass, more specifically BMD. The use of BMD as a surrogate measure of determining the risk of developing a fragility fracture has been validated, and is used by health care professionals to determine an individual's risk of developing osteoporosis [52]. It is most clinically relevant to measure BMD with DXA at the femoral neck (hip fracture) and spine (vertebral compression), as these are common sites of fractures [53] associated with marked morbidity and premature death [54]. Current research has demonstrated that the gold standard for determining bone strength, and subsequently fracture risk, is through

the combination of BMC and BMD by DXA, as well as microstructural properties from CT, in excised femurs from human cadavers [55]. However, DXA is still primarily used in clinical settings, as it is cost-effective with minimal exposure to radiation [56].

Considering that nutrition can modulate bone health, it may be important to establish links between a high fat diet and overall bone health. Studies examining the impact of a high fat diet on bone health often use animal models, more specifically rodents, as it is difficult to measure the true properties of bone (i.e. bone strength) in humans. Bone strength in rodents is determined by fracturing the excised bone, and obtaining its 'peak load', which is the maximum force a bone can withstand before fracturing. Peak load is a surrogate measure of bone strength, a reason why rodent models are often used in intervention studies. It has been shown that a high fat diet may impact trabecular bone more than cortical bone [43, 57, 58], which will be discussed further in subsequent sections. This may be due to the high metabolic activity in trabecular bone [59], with greater nutrient delivery, compared to cortical bone. Furthermore, weight-bearing bones may respond to a high fat diet to an even greater extent compared to non-weight-bearing bones, due to a higher rate of bone turnover [60, 61]. Therefore in rodent models, it is particularly important to examine the impact of hindlimb bones such as the femur and tibia, as these are considered weight-bearing [62]. It is also important to investigate whether diet can modulate bone at different skeletal sites, particularly the spine, which is known to be rich in trabecular bone. The effect of a high fat diet on rodent bone health is further discussed in section 2.2.1.

### 2.2.1. Effect of high fat diet consumption on bone quantity, quality, strength, and fatty acid composition in rodents

As previously described, bone health is a measure of bone strength, which is often determined by bone quantity (mineral content) and quality (structure). It is the investigation of these three components that will provide the greatest understanding of overall bone health. However, when determining the impact of a high fat diet on rodent bone health, there are only a handful of studies in the literature, and only one has investigated all three of these important outcomes.

A study by Lanham et al. investigated the impact of a high fat diet (22% fat by weight – 18% lard, 4% soya oil) on femoral density, structure and strength of growing C57BL/6 mice [58]. A strength of this particular study was that the levels of protein, vitamins, and minerals were increased on an energy basis to account for the elevated levels of fat in the high fat diet [58]. It is important to consider that the higher fat content in a high fat diet subsequently increases overall energy provided, as it will change the relative energy contribution of other nutrients that are important for bone health (i.e. calcium, vitamin D, protein). When energy content of a diet is higher per kg of diet, it is important to proportionately adjust for micronutrients, as well as protein content, to account for the higher amount of energy provided per kg of diet. This is to ensure that the proportion of vitamins and minerals, as well as protein, is similar to that of the control diet and thereby enables investigators to directly evaluate the effects of the fat in the diet on overall bone health.

Mice were fed the high fat or control (5% corn oil by weight) diet from weaning to 7.5 months of age, at which point femoral density and structure were determined



using micro-computed tomography ( $\mu$ CT), and strength using a materials testing system. Femur density was greater in both males and females fed the high fat diet compared to mice fed the control diet [58], which may have been a result of excess nutrient consumption. Lanham et al. also found differences in trabecular, but not cortical structure in femurs of mice fed the high fat diet [58]. Bone volume and trabecular spacing were greater in females fed the high fat diet, but not males, when compared to mice on a control diet. However, despite these changes in femoral density and trabecular structure, there were no differences in peak load (the maximum load a bone can withstand before fracture – measure of bone strength) at the femur midpoint [58]. Considering the dietary adjustment of nutrients (protein, vitamins, minerals) in the high fat diet, it is possible to conclude that a high fat diet containing 22% fat by weight may not be detrimental to overall bone health. However, this study only examined the impact of a high fat diet on one skeletal area. It may be important to continue investigations in other skeletal sites, such as vertebrae (rich in trabecular bone), to determine whether effects are site-specific. Moreover, bone strength was only measured at the femur midpoint, which is a site that is rich in cortical bone. Considering that cortical bone structure did not change, but trabecular bone did by greater trabecular spacing, it may be important to determine bone strength at sites rich in trabecular bone (e.g. femur neck, vertebrae).

Another study investigated whether the quality of dietary fat had differing effects on bone quantity and strength. Lau et al. fed 1.5 month old male Sprague Dawley rats standard chow, or one of three different high fat diets containing different types of fat as the fat source. Each of the high fat diets contained 20% total fat by weight, and were

enriched in either: saturated fat (18% coconut oil, 2% soybean oil by weight); *n*3 polyunsaturated fat (20% flaxseed oil by weight); or *n*6 polyunsaturated fat (20% safflower oil by weight) [63]. After approximately 2 months of feeding, BMC and BMD of excised femurs were determined using DXA, and peak load at the femur midpoint was measured with a materials testing system. There were no differences in femoral BMC or BMD between groups [63]. Furthermore, rats fed the diets high in *n*3 and *n*6 polyunsaturated fats had greater peak load at the femur midpoint when compared to chow-fed rats, even when adjusted for body weight. However, there were no differences in femur midpoint peak load in rats fed the high saturated fat diet [63], which is consistent with findings from the study by Lanham et al. [58]. Therefore, this study by Lau et al. demonstrates that a diet high in saturated fat does not alter BMC, BMD, or femur strength after 2 months of feeding [63]. The findings that a high saturated fat diet did not contribute to differences in BMC and BMD in this study [63] are inconsistent with those from the study by Lanham et al. [58], which showed increased femoral density [58]. Differences in study findings may be due to the duration of dietary intervention, as mice in the study by Lanham et al. [58] were fed the high fat diet for a longer period of time compared to the duration of feeding in the study by Lau et al. [63], where those mice may have adapted to excess nutrient consumption with greater bone accretion. The quality of saturated fat may have played a role in differences in study findings, where the study by Lau et al. [63] used coconut oil (mainly medium-chain fatty acids), whereas the dietary intervention in the study by Lanham et al. [58] incorporated lard (mainly long-chain fatty acids). Medium-chain fatty acids are readily absorbed in the stomach, entering the portal system, and are preferentially oxidized compared to

long-chain fatty acids [64]. In rodents, there are fewer medium-chain fatty acids compared to long-chains found in triglycerides [65]. The preferential oxidation of medium-chain fatty acids, as provided in the dietary intervention through coconut oil, may have contributed to the lack of increased bone mineral content in the study by Lau et al. [63]. Elevated bone mineral density as an effect of consumption of a high saturated fat rich in long-chain fatty acids (provided by lard) in the study by Lanham et al. [58], may have been a result of increased storage of these fatty acids in bone, potentially promoting bone modelling. Nonetheless, differences in study findings may have been mediated by the quality of dietary fat, rather than the quantity. Furthermore, as previously discussed, it may be important to determine whether effects are consistent amongst each bone type, especially considering the high metabolic activity of trabecular bone [59], which may render it more sensitive to changes in diet compared to cortical bone.

A study by Ward et al. investigated the impact of a Western-style diet on bone quantity and strength in femurs of Fischer 344 rats [66]. The Western-style diet designed in this study was high in fat (20% corn oil by weight) and sucrose, with low levels of calcium, vitamin D, folic acid, and fibre. Rats, approximately 2 months of age, were fed the Western-style diet or control diet for approximately 4 months. Bone mineral content (BMC) was measured using the ash technique [66], where femurs were dried and ashed in a furnace before mineral content was calculated ( $[(\text{dry mass} - \text{ash mass}) \times 100 / \text{dry mass}]$ ) [67]. Femur midpoint peak load determined with a materials testing system [66]. BMC was significantly lower after approximately 4 months of feeding, as was femur midpoint peak load. It is difficult to truly attribute these detrimental effects on bone to

the level of fat in the diet. Femurs may be weaker due to the lack of sufficient levels of calcium and vitamin D, which are known to be important for bone health [68, 69].

Parhami et al. demonstrated that lifelong consumption of a high saturated fat diet can negatively impact the quantity of bone [70]. In this study, 1 month old male C57BL/6 mice were provided a high saturated fat diet (16% total fat by weight; approximately half from cocoa butter) for 4 and 7 months. The diet was intended to induce atherosclerosis, with additional cholesterol (1% by weight), which was mixed with standard mouse chow (75% chow by weight). Both BMC and BMD of femurs and fourth lumbar vertebra (LV4) were measured using quantitative CT at 4 and 7 months of age [70]. At 4 months of age, femoral BMD in 3 of 8 scanned regions was lower due to high fat feeding. Later in adulthood at 7 months of age, femoral BMD was lower in 6 of 8 sites, with lower BMC in the femur and LV4 of high fat fed rats [70]. This study is the only one to demonstrate that a diet high in saturated fat can lead to lower bone mineral. This is interesting as the level of fat used in the study by Parhami et al. [70] was lower than that implemented in those previously described ([58, 63, 66]). Furthermore, despite using the same experimental model (C57BL/6) and studying mice at a similar age (7 and 7.5 months old) as the study by Lanham et al. [58], the lowering of BMC and BMD in the study by Parhami et al. [70] are conflicting as Lanham et al. found an overall increase in femoral density [58]. It is difficult to directly compare and contrast these findings as the instruments used to determine bone quantity were not the same (quantitative CT versus  $\mu$ CT), and will produce different measurements due to the nature of the machine. It is clear that future research is needed in this area considering the discrepancies in study findings.

Two studies by Cao et al. examined the effects of a high fat diet in a growing model, using 1.5 month old male C57BL/6 mice [43, 57]. Mice were fed a high saturated fat diet (23.6% total fat by weight – 20.7% lard, 2.9% soybean oil) for 3.5 months, and cortical and trabecular structure of tibias and femurs were determined using  $\mu$ CT [43, 57]. Though no differences were examined in cortical bone, mice fed the high fat diet had increased trabecular separation and lower trabecular number, despite being obese [43, 57]. These results indicate the tibias and femurs from high fat fed mice were more porous compared to animals fed a control diet, creating a weaker structure, which can contribute to a greater risk of fracture. Furthermore, these studies [43, 57] provide evidence that increased loading due to higher body weights such as in obesity may not necessarily be protective of overall bone health. These findings are in accordance with those from Lanham et al., which demonstrated that femoral trabecular structure, but not cortical structure, was impacted due to consumption of a high fat diet [58]. It may be that trabecular bone is more sensitive to dietary interventions, considering it is more metabolically active. However, it is not known whether these alterations in trabecular structure translate to changes in bone strength in skeletal sites rich in trabecular bone, such as the femur neck and vertebrae. Further investigation is required in this area, as trabecular structure is a predictor of fracture risk in osteoporosis [71, 72].

The studies discussed above demonstrate that high fat diet consumption can alter bone mineral [70], structure [43, 57] and strength [66] in rodents, which may ultimately alter the risk of fracture. Whether this is due to direct effects of fatty acids present in bone or due to a secondary mechanism is not known. It is well established that dietary fat intake has the ability to alter the fatty acid composition of cortical bone. In general,

the composition of fatty acids in the dietary fat consumed reflects the fatty acid composition of cortical bone, as determined by animal models [63, 73, 74]. Most studies that have investigated the impact of a high fat diet on fatty acid composition in bone have focused on diets high in *n*3 and *n*6 polyunsaturates (PUFAs) [74, 75]. However, the effect of a high saturated fat diet on bone fatty acid composition is not well studied. Whether elevations saturated fatty acids in cortical bone results in functional changes (i.e. reduced bone strength) remains to be established. This is an important area of discovery considering the typical Canadian diet, and supporting evidence from animal models that a diet high in saturated fat can lead to lower bone quantity [63, 66, 70] and altered structure [43, 58].

It is evident that a high fat diet can modulate bone health; however there are discrepancies in study findings that make it difficult to make comparisons. Although most studies measure bone mineral, the instruments amongst studies have all varied. Consistency in methods and instruments used amongst studies may facilitate comparisons. Furthermore, studies that have investigated the impact of high fat diet consumption on bone structure have shown that trabecular bone is preferentially altered, with no changes in cortical bone [43, 57]. However, whether trabecular bone is altered at various skeletal sites remains to be determined. In addition, the current studies have only measured bone strength at sites rich in cortical bone. Considering any structural alterations (i.e. increased trabecular spacing [58]) due to high fat diet consumption appear to be restricted to trabecular bone, bone strength should be measured at trabecular-rich sites (e.g. femur neck, vertebrae). Only one study by Lanham et al. [58] has appropriately adjusted for the level of protein, vitamins, and minerals in the high fat

diet. Further investigation in this area should adjust for the level of these important nutrients to appropriately attribute any observed effect in bone, to the level of fat in the diet, and not inadequate intake of these nutrients. Further research in the area of bone fatty acid composition and its impact on bone strength is needed. This would provide a greater understanding of the functional importance of changes in bone fatty acid composition. Lastly, no study to date has investigated bone quantity, quality, strength, and fatty acid composition in one study. This would provide a more comprehensive understanding of overall bone health.

### **2.3. Impact of high fat diet consumption on plasma hormones**

As discussed in section 2.1, consumption of a high fat diet in rodents is known to increase overall adiposity [49, 50], which is associated with increased levels of circulating pro-inflammatory cytokines [76] released from adipocytes. A high fat diet may lead to an increase in visceral adipose tissue, whose release of pro-inflammatory cytokines can lead to the development of morbidities such as non-alcoholic fatty liver disease. Liver disease is manifested by increased delivery of pro-inflammatory cytokines and fatty acids, as visceral adipose tissue drains directly into the portal system. In addition, the presence of certain pro-inflammatory cytokines can also impact bone health. More specifically in high levels, these cytokines are known to increase bone resorption [77] and inhibit bone formation [78]. Pro-inflammatory cytokines, chemokine attractants, and associated hormones will be discussed in terms of their relation to high fat feeding and increased fat mass, as well as bone health, in sections 2.3.1 – 2.3.6.

### 2.3.1. Leptin

Leptin is an adipokine released by white adipose tissue [79] that exerts its effects on body composition and bone mass. Circulating leptin is positively correlated with fat mass [80], is an appetite suppressor, and known regulator of body weight [81]. It is well established that a high fat diet leading to obesity causes drastically increased circulating levels of leptin [80]. Greater energy intake from consumption of a high fat diet may lead to increased fat storage, thereby elevating leptin concentrations above normal circulating levels. Knowing that leptin is an appetite suppressor, it is clear the leptin functioning is impaired in obesity. High circulating levels of leptin without normal leptin functioning is known as leptin resistance [82]. Leptin resistance occurs when there is a lack of a response (i.e. suppressed appetite) after provision of endogenous and /or exogenous leptin [83]. The concept of leptin resistance has been greatly debated amongst investigators and the mechanisms to which this may occur are not clearly defined. Some believe leptin resistance occurs as a result of signalling or transport defects. More specifically, alterations in signalling through leptin receptor b (LRb) in the arcuate nucleus [84], impaired transport from plasma across the blood-brain barrier to reach a LR [85], or promotion of processes that inhibit LRb signalling (e.g. inflammation) [86]. Current research in the area is testing the conditions in which these hypotheses are more likely to occur.

There is controversy at present in regards to the literature on the effects of leptin on bone. Studies are reporting that leptin has either antiosteogenic or osteogenic effects on bone, which has been studied extensively in *in vitro* and *in vivo* models, as well as clinical trials. The antiosteogenic impact of leptin is thought to act through the central



relay of leptin, specifically indirectly by binding to the LR on the hypothalamus. This stimulates the release of norepinephrine, which binds to the  $\beta$ -adrenergic receptor on the osteoblast, inhibiting osteoblastic function in mice [87]. This antiosteogenic effect was first discovered in mice with leptin (*ob/ob*) or leptin-receptor (*db/db*) deficiencies, which have characteristic high bone mass phenotypes with the inhibitory effects of leptin diminished [78]. Furthermore, central regulation of leptin was proven to be antiosteogenic as leptin infusion (intracerebroventricular – stimulates central relay of leptin) in *ob/ob* mice for 28 days lowered bone mass, as discovered by histology of vertebrae [78].

The osteogenic impact of leptin occurs outside of the central relay, where circulating leptin can exert its effects directly on bone. In contrast to the antiosteogenic effects of leptin when centrally regulated, peripheral leptin is thought to have potential anabolic effects on bone, specifically through upregulation of bone formation. One study that provided exogenous leptin to *ob/ob* rodents discovered that leptin increased bone mass following administration [88]. This evidence suggests that leptin secreted from adipose tissue can modulate bone formation. Furthermore, greater fat mass attributed to a high fat diet, producing even greater levels of leptin may have a more potent anabolic effect on bone.

It is known that obese individuals have high BMD compared to individuals of healthy weight [89], due to increased mechanical loading from excess body weight. Leptin resistance may also contribute to this increase in BMD in obesity. With high circulating levels of leptin, there is a greater chance for leptin to bind to its receptor on bone, which consequently has osteogenic effects. Furthermore, the mechanisms in which

leptin resistance occur, stem from the brain where the central relay of leptin. As previously mentioned, the effects of leptin from the central relay seem to have antiosteogenic effects. Therefore, it may be that leptin resistance occurs in the brain, preventing the central relay of leptin and its antiosteogenic effects. However, this relationship is a speculation and remains to be determined. Despite discrepancies in the literature, it is evident that leptin has potent effects on bone. Any changes to circulating levels of leptin due to diet, increases in fat mass, or disruptions in central regulation of leptin, will subsequently impact bone homeostasis.

### 2.3.2. Tumour Necrosis Factor-Alpha (TNF- $\alpha$ )

TNF- $\alpha$  is a pro-inflammatory cytokine released by a number of different cells, including macrophages, lymphocytes, and adipose tissue [90]. High circulating levels of TNF- $\alpha$  is an indicator of low-grade inflammation, commonly found in obesity [91]. TNF- $\alpha$  has been shown to be secreted progressively more by adipose tissue as fat mass increases [92]. Therefore, high fat diet-induced increases in fat mass will also contribute to higher levels of TNF- $\alpha$ . At the tissue level, higher circulating levels of TNF- $\alpha$  may contribute to dysfunction of the tissues affected. For example, TNF- $\alpha$  is known to reduce insulin sensitivity in adipose tissue when found in high amounts, which can lead to insulin resistance [93]. TNF- $\alpha$  responds in accordance to changes in body composition and fat distribution, and is a potent contributor to the development of other health complications especially in situations of chronic inflammation [93].

TNF- $\alpha$  not only contributes to chronic inflammation, but has detrimental effects on bone health as well. TNF- $\alpha$  down-regulates production of osteoblasts and promotes

production of osteoclasts, favouring bone resorption [94]. There are multiple ways in which TNF- $\alpha$  inhibits bone formation, which will be discussed in brief. TNF- $\alpha$  inhibits type I collagen production [95], lowers levels of osteocalcin and decreases sensitivity to vitamin D, and inhibits normal function of alkaline phosphatase (required for bone mineralization). In addition, TNF- $\alpha$  can prevent osteoblast differentiation [95], which would also contribute to slower bone formation and promoting greater bone resorption. TNF- $\alpha$  also acts in a number of ways to promote bone resorption. TNF- $\alpha$  increases osteoclastogenesis, mediated by NF- $\kappa$ B, by upregulation of RANK expression [94].

From the review of the literature, it is clear that TNF- $\alpha$  is a contributor of low-grade chronic inflammation as seen in individuals with elevated fat mass (e.g. obesity). Additionally, its effects can be deleterious at the tissue level, particularly in bones by promoting bone resorption. This may eventually contribute to weaker bones as with greater rates of bone resorption. A high fat diet per se, does not directly promote TNF- $\alpha$  production, however when paired with a sedentary lifestyle, greater fat mass can accumulate. This may contribute to a higher circulating level and increased expression of this pro-inflammatory cytokine, which can ultimately lead to greater health problems if not dealt with.

### 2.3.3. Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory cytokine with multiple functions, and like TNF- $\alpha$  is secreted by a number of different cells, including adipocytes. Levels of IL-6 are correlated with overall fat mass [96], which is secreted in highest amounts by visceral fat (compared to subcutaneous fat) [97, 98]. It has also been established that IL-6 secreted by

visceral adipose tissue is released into the portal vein and can contribute to systemic inflammation in obese women [99]. A high fat diet may contribute to accumulation of fat mass (especially visceral), leading to high circulating levels of IL-6 and subsequently lead to systemic inflammation.

IL-6 is also secreted within bone, particularly by osteoblasts [77, 100], however its role does not favour bone formation. Bone resorption appears to be indirectly mediated by IL-6 through its receptor, IL-6R, as increasing osteoclast production has been observed *in vitro* [101, 102]. IL-6 also stimulates and enhances the effects of other inflammatory cytokines, such as TNF- $\alpha$  when present, which further contributes to increased osteoclastogenesis [103] and bone resorption. Therefore in the presence of a high fat diet, greater fat mass accumulation and subsequent increases in circulating levels of IL-6 may also have detrimental effects on bone remodelling, in the favour of bone resorption.

#### 2.3.4. Monocyte chemoattractant protein-1 (MCP-1)

MCP-1 is a chemokine responsible for the translocation of monocytes during inflammation [104]. The effects of MCP-1 with increased adiposity, as can be induced with a high fat diet, can promote chronic low-grade inflammation often observed in obesity [105]. Low-grade inflammation is thought to be, in part attributed to increased macrophage infiltration of adipose tissue, mediated by MCP-1 [105]. It is recognized that levels of MCP-1 in plasma and adipose tissue are associated with the level of adiposity, and are secreted in greater amounts by visceral adipose tissue compared to subcutaneous

[42]. Low-grade inflammation can not only contribute to insulin resistance and atherosclerosis, but can also negatively impact bone health.

MCP-1 is not always found in healthy bone, but is preferentially expressed in conditions of inflammation [106, 107] for recruitment of monocytes. Studies using a cell culture model of human osteoblasts have demonstrated that osteoblasts secrete MCP-1 in presence of a pro-inflammatory cytokine, IL-1 [108]. Additionally, it has also been shown that MCP-1 is present during bone remodelling, as discovered in mice following tooth eruption (where bone formation and resorption occur simultaneously) [104]. Bone remodelling has also been shown to be induced by MCP-1, through osteoclast and precursor monocyte recruitment to the site of remodelling in cell culture. Parathyroid hormone (PTH) stimulates the release of MCP-1 by osteoblasts, which ultimately exert effects on osteoclast function, and can also upregulate osteoclastogenesis. In situations where PTH is transiently elevated in cell culture, MCP-1 can help exert anabolic effects, as bone formation would still exceed bone resorption. However, when PTH levels are elevated for extended periods of time, MCP-1 can have catabolic effects on bone, favouring resorption over formation [109]. Therefore, continuous stimulation and secretion of MCP-1 has the potential to tilt bone remodelling in favour of resorption, which can be detrimental to overall bone health if sustained over time.

#### 2.3.5. $17\beta$ -Estradiol

Estradiol is a form of estrogen secreted by the ovaries and is a major regulator of both body composition and bone mass [110, 111]. During menopause and subsequently after, women experience changes in body composition, particularly increases in fat mass

[110]. Moreover, post-menopausal women accumulate more fat mass subcutaneously and visceraally, which is accompanied by lower rates of fat oxidation [112, 113]. Though total fat mass is known to increase, there is also a marked increase in abdominal adiposity [112, 113]. Furthermore, post-menopausal women who receive hormone replacement therapy (HRT) have reduced fat mass accumulation compared to women who do not receive HRT [114]. Ovariectomized rats (estrogen deficient), have characteristic increases in visceral adiposity that can be diminished with administration of estradiol [110]. The effects of estradiol on body composition and fat mass distribution are not only true for women, but are also evident in men (and rodents) with aromatase deficiency. Aromatase deficiency prevents the production of estrogen in men and knockout animal models, leading to a stereotypic phenotype of increased adiposity compared to normal men [115, 116]. Therefore, it is evident that normal circulating levels of estrogen are important for maintaining lipid homeostasis and distribution of fat, which can be easily disrupted when levels become too low [112].

Estradiol and other estrogens have powerful effects on bone remodelling, with receptors located on both osteoblasts and osteoclasts [117]. Estradiol acts mainly through estrogen receptor  $\alpha$  (ER $\alpha$ ) on osteoblasts and osteoclasts [118], each receptor with a separate function. ER $\alpha$  located on osteoblast precursors promote formation of cortical bone, while ER $\alpha$  located on osteoclasts promote trabecular bone formation [117, 119]. Estradiol has anabolic effects on bone by promoting osteoclast apoptosis, and decreasing osteoclastogenesis for overall decreased resorption. Not only is resorption decreased, bone formation rate is further improved [111].

It is clear that normal circulating levels of estradiol is imperative for promoting healthy fat distribution, and maintenance of bone health. In situations where levels of estradiol deviate from normal ranges, such as in menopause, subsequent changes in fat mass distribution and bone health occur as a result. Given the increased consumption of a Western diet containing high levels of saturated fat, it is important to understand the impact of fat on estradiol concentrations and its impact on body composition and bone health.

#### 2.3.6. Progesterone

Progesterone is another female sex hormone known to exert its effects on a number of tissues and organs, including the uterus, adipose, and bones. The effects of progesterone on body composition and fat mass distribution are not as well defined as estrogen, however some studies have determined that progesterone may be involved in lipid synthesis. Female rats given progesterone gained more weight than rats not exposed to progesterone [120], which translated into greater total body fat [121]. Additionally, women who used a contraceptive made of progesterone also had experienced weight gain and increases in the level of whole body fat mass [122]. Administration of progesterone to 3T3-L1 preadipocytes led to differentiation to adipocytes [123]. Other studies *in vitro* found that progesterone stimulates the activity and increases gene expression of fatty acid synthase, an enzyme responsible for long-chain fatty acid synthesis [124]. There is some evidence that progesterone leads to the characteristic gynoid (lower body) distribution of adipose tissue in women. Administration of progesterone in female rats also increased gene expression of lipogenic enzymes within inguinal adipose tissue [125], which may in

part contribute to lower body adipose storage and distribution. Similar to estrogen, absence of progesterone such as during menopause, can lead to visceral fat storage [113], which has multiple metabolic consequences.

As previously mentioned, progesterone has anabolic effects on bone which become diminished during menopause. Though it does not have a particularly potent effect on bone like estradiol, progesterone has an important function in regulating bone formation [126]. Progesterone receptors are evident in human osteoblast-like cells [127], and when supplemented in primary culture of osteoblasts, progesterone increases proliferation in human and rat cells [128, 129]. The effects of progesterone on reversal and prevention of bone loss has been studied extensively in medications combined with estrogen. Although not commonly used as a medication to treat rapid bone loss, some studies have reported that progesterone alone is effective in slowing the rate of bone loss in post-menopausal women [130, 131].

Progesterone, in normal physiological levels, is an important mediator of non-threatening fat storage (gynoid) and bone formation. When levels are low, its role in regulating these important functions in adipose tissue and bone become disrupted as described above. It is unclear whether a high fat diet has the potential to alter levels of progesterone in women, which may be fundamentally important to determine considering the typical Canadian diet.

### **Chapter 3.0: Nutritional Programming**

While lifestyle factors such as diet and physical activity are known to modulate risk of chronic disease development [132-135], less understood is the role of maternal



diet in chronic disease development among offspring. However, the intrauterine environment is a critical determinant of childhood and subsequent adult health as metabolic perturbations in utero largely impact body weight regulation and fetal skeletal development [136]. Therefore, an overall unhealthy or nutritionally unbalanced maternal diet during pregnancy can alter offspring nutrient availability that can ultimately impact offspring growth and development. More specifically, it has been discovered that long lasting changes in offspring phenotype may occur as a result of a poor maternal diet that may contribute to increased risk of disease in adulthood. These long lasting changes in offspring phenotype are a result of ‘nutritional programming’- which is the effect of a food or a food component exerted on offspring when exposure occurs in early life.

One particular event brought about further investigation of nutritional programming, which was the Dutch winter famine (November 1944 – April 1945) occurring in the last winter of World War II [137]. The birth cohort derived from the Dutch winter famine – that resulted from a harsh winter limiting agricultural production as well as the Germans limiting food supply to regions of Holland - has provided a unique opportunity to investigate the true effects of maternal diet on offspring health and development, being a true ‘natural experiment’ [137]. The birth cohort was derived from mothers deprived of food before, during, and prior to pregnancy during the Dutch winter famine. Studies over the past two decades have found associations between poor adult health, such as the increased risk of developing chronic diseases, in offspring whose mothers were exposed to the Dutch winter famine [138, 139]. This has provided framework for current investigations in the topic of nutritional programming due to maternal *under-nutrition*, which is being extensively studied in animal models.

Though it is imperative that maternal under-nutrition be investigated, especially considering the numerous countries in poverty, it is also of particular importance to investigate the impact of maternal *over-nutrition* on offspring health. This is important given the rise in obesity worldwide and greater food accessibility. There is evidence in animal models to support that offspring from mothers fed vitamins or multivitamins had long-lasting changes in overall health [140, 141]. More specifically, mothers exposed to the multivitamin had male offspring that were obese and hyperphagic, with glucose intolerance [140, 141]. Given this hypothesis of the long-term consequences on offspring health due to maternal over-nutrition, it is thought that obesity is also programmed in utero due to maternal diet. Recent evidence has discovered genomic alterations through DNA methylation and changes in chromatin structure through histone modification in offspring due to suboptimal maternal diet [58]. This creates changes in DNA expression, which is one of the proposed reasons for alterations in offspring phenotype. A particular example of DNA methylation is shown in a study by Chen et al., which examined the effects of maternal high fat diet consumption on fetal bone development [142]. Fetal mice from mothers fed a high fat diet during pregnancy had delayed bone development at embryonic day 18.5, despite typical intramembranous ossification occurring at embryonic day 15 in mice [142]. This effect may be facilitated by methylation of HoxA10, an important modulator of PPAR $\gamma$  (transcription factor for adipogenesis) and Runx2 expression [142].

It is also important to understand whether there are particular periods in which offspring are most vulnerable to an unhealthy maternal diet. Evidence from the Dutch winter famine describes that exposure of famine in early gestation was associated with

the development of a greater number of chronic diseases (e.g. 5 diseases – breast cancer, obesity, coronary heart disease, glucose intolerance, atherogenic lipid profile) compared to individuals exposed to the famine in late gestation (e.g. 1 associated disease – glucose intolerance) [143]. Therefore, although any nutritional insult during pregnancy can have long-lasting impacts, it may be that early gestation is the critical period in which offspring are most vulnerable to maternal diet. This is because early gestation, or the first trimester, is the time in which the fetus is rapidly growing, and organs are developing [144].

It is essential to explore the effects of nutritional programming in both male and female offspring, however it may be of particular interest to look closely at the effects in female offspring as existing literature already identifies maternal health as a predictor of infant health [139, 145-147]. Permanent changes in female offspring genotype may also have the ability to be passed to subsequent generations during future pregnancy. Therefore, it is important to study female offspring to understand the consequences of poor maternal health and nutrition to protect the health of future offspring. Current knowledge regarding the role of maternal nutrition prior to, and during pregnancy and lactation on offspring body composition, bone development, and circulating hormones will be discussed in sections 3.1-3.3.

### **3.1. Effect of maternal high fat diet consumption on body composition in female rodent offspring**

It is well established that elevated fat mass, especially visceral fat, is a risk factor for the development of chronic diseases such as cardiovascular disease [46] and insulin

resistance [45, 148]. With the evidence that maternal diet programs offspring health [140, 141], it is important to determine whether offspring body composition is altered as a result of a maternal high fat diet. A number of studies have investigated the effects of maternal diet on offspring body composition in various conditions [17, 44, 149, 150]. Some studies have only focused on the impact of maternal diet, exposure of maternal diet occurring solely in utero [17, 150], while others have investigated whether exposure to maternal diet *postnatally* (lactation and/or post-weaning) has greater effects on offspring health [44, 149, 151]. Furthermore, the *age* or *time of exposure* of maternal diet, as well as the age at which offspring were studied, have also differed amongst studies. These individual aspects regarding the impact of a maternal high fat diet on female offspring body composition are highlighted in Table 1, and will be explored in detail below.

Adverse effects of maternal high fat feeding are evident in female offspring in early life. However only two studies to date have investigated the impact of maternal high fat feeding on offspring body composition in early life. Krasnow et al. investigated whether various durations of maternal exposure to a high fat diet (35% fat by weight - 32% lard, 3% soybean oil) had different impacts on body composition in newborn C57BL/6 female offspring mice [150]. The second study was by Sun et al., which investigated the impact of feeding Sprague Dawley rats the same high fat diet used in the study by Krasnow et al. (35% fat by weight - 32% lard, 3% soybean oil) throughout pregnancy and lactation on female offspring body composition at weaning. Offspring were cross-fostered at birth (i.e. females from dams fed high fat diet were fed by dams fed control diet during lactation) [17], so that the impact of high fat diet exposure in utero could be studied separately from the diet consumed while suckling. This was done to

decipher whether differences detected at weaning were due to prenatal or postnatal diet. [17].

Regardless of the length of exposure of mothers to the high fat diet, all offspring from these mothers had overall lower whole body lean mass [17] and greater fat mass compared to offspring from mothers fed a control diet [150]. However in the study by Sun et al., offspring from mothers fed a high fat diet, cross-fostered to mothers fed chow diet did not have differences in body composition [17]. Rather, a postnatal high fat diet caused greater overall subcutaneous and visceral fat (as % of total fat), with lower lean mass, in female Sprague Dawley rat offspring at postnatal day (PND)10 and PND21 compared to females fed chow postnatally [17]. Therefore, there is evidence that a maternal high fat diet can alter female offspring body composition at birth, by increasing fat mass and fat:lean mass ratio [150]. However, once females are exposed to a healthier diet during suckling, the maternal high fat diet does not seem to have an impact on offspring body composition [17]. Despite this evidence, further research in the area should be investigated to develop definitive answers on the impact of a maternal high fat diet on female offspring body composition in early stages of life.

The influence of a maternal high fat diet on female offspring body composition at adulthood has also been investigated. Overall, 3 of the 4 studies that examined this in female offspring at adulthood (4,5 and 9 months of age) found that fat mass and fat:lean mass ratio were increased as a result of a maternal high fat diet [44, 149, 151]. These studies used similar maternal high fat diets, which contained either 24% total fat by weight (21% lard, 3% soybean oil [44, 151]) or 22% total fat by weight (18% lard, 4% soya oil [149]). In the study by Howie et al., females Wistar rats from mothers fed a high

fat diet (24% total fat by weight - 21% lard, 3% soybean oil) throughout life, plus during pregnancy and lactation, had similar increases in fat:lean mass ratio than females from mothers fed the high fat diet solely during pregnancy and lactation [44]. The findings from this study confirm that maternal consumption of a high fat diet can impact female offspring body composition in adulthood. Furthermore, this study provides evidence that *timing* of the maternal high fat diet rather than *duration*, is a more potent modulator of female offspring body composition in adulthood (~5 months of age) [44]. However, the study by Connor et al. contradicts this conclusion that *timing* of maternal high fat feeding dictates changes in female offspring body composition in adulthood [151]. Connor et al. also found that female Wistar rat offspring from mothers fed a high fat diet throughout pregnancy and lactation had higher fat:lean mass ratio, but only in females fed a post-weaning high fat diet [151]. In the study by Howie et al., this finding occurred regardless of post-weaning diet (control *or* high fat diet) [44]. This is particularly interesting as both studies used the same animal, high fat diet, study design, and method for determining whole body fat mass (as shown in Table 1) [44, 151]. The results from the study by Connor et al. suggest that high fat diet consumption post-weaning may impact female offspring body composition in adulthood (~4 months of age) greater than a maternal high fat diet [151]. This conclusion is consistent with the findings in the study by Sun et al. [17]. However, reasons as to why study findings of female offspring body composition differ in the studies by Howie et al. [44] and Connor et al. [151], despite having similar experimental designs, are unknown.

The study by Elahi et al. confirms the finding of increased fat mass in female C57BL/6 mice at adulthood (9 months of age) from mothers fed a high fat diet (22% total

Table 1. Summary of the literature regarding the impact of a maternal high fat diet on female offspring body composition in rodents

Species	High fat diet (% fat by weight)	Duration of maternal diet	Age of offspring and diet	Methods	Female offspring body composition	Reference
C57BL/6 mice	<b>34.9% total fat</b> 31.7% lard 3.2% soybean oil	4, 8, or 11 weeks	PND0	NA	↓ Lean mass ↑ Visceral fat	Krasnow et al.[147]
Sprague Dawley rats	As above	Pregnancy + lactation	PND10 and 21 ( <b>CON or HF</b> )	NMR	<u>Post-weaning <b>HF</b> diet:</u> ↓ Lean mass ↑ Fat mass	Sun et al.[17]
Wistar rats	<b>24% total fat</b> 21% lard 3% soybean oil	<b>1) Lifetime</b> (23 weeks, pregnancy + lactation) <b>2) Pregnancy +</b> lactation	~5 months ( <b>CON or HF</b> )	DXA	<u>Post-weaning <b>CON</b> and <b>HF</b> diet:</u> ↑ Fat:lean ratio ↑ BMC	Howie et al.[44]
Wistar rats	As above	Pregnancy + lactation	~4 months ( <b>CON or HF</b> )	DXA	<u>Post-weaning <b>HF</b> diet:</u> ↑ Fat:lean ratio	Connor et al.[148]
C57BL/6 mice	<b>22.5% total fat</b> 19.5% lard 3% soybean oil	1.5 months, pregnancy + lactation	3.5 or 6.5 months ( <b>All CON</b> )	DXA	↓ Fat mass (3.5 months) ↓ BMC (3.5, 6.5 months)	Devlin et al.[149]
C57BL/6 mice	<b>22% total fat</b> 18% lard 4% soya oil	1.5 months, pregnancy + lactation	9 months ( <b>CON or HF</b> )	Excised fat pad weights	<u>Post-weaning <b>CON</b> and <b>HF</b> diet:</u> ↑ Fat mass	Elahi et al.[146]

PND – Postnatal day; CON – Control diet; HF – High fat diet; NA – not available; NMR – Nuclear magnetic resonance; DXA – Dual-energy x-ray absorptiometry

fat by weight - 18% lard, 4% soya oil) for 1.5 months plus during pregnancy and lactation [149]. Similar to the study by Howie et al. [44], this overall whole body fat mass remained higher in females from mother fed a high fat diet that also consumed a high fat diet post-weaning [149]. This study contradicts the conclusion from Connor et al. [151], and suggests that there may be programming in female offspring body composition at months of age due to maternal consumption of a high fat diet [149]. A similar study in C57Bl/6 mice by Devlin et al. [152] however, contradicts the study findings by Elahi et al. [149].

The study by Devlin et al. is the only one to show lowering of fat mass in female offspring at adulthood (3.5 months of age), indicating no programming effect from maternal consumption of a high fat diet (22.5% total fat by weight – 19.5% lard, 3% soybean oil). However, female offspring were also studied until 6.5 months of age, at which point there were no differences in fat mass, indicating no programming effect from the maternal high fat diet [152]. These findings differ greatly from that of Elahi et al. [149], despite having the same experimental model, study design, and similar maternal high fat diet [149]. Females in the study by Devlin et al. may have lower fat mass at 3.5 months of age due to a mismatch in caloric intake from the high fat diet consumed during gestation and suckling, to post-weaning control diet [152]. Female offspring may have initially had greater fat mass than females from mothers fed control diet, as they may have adapted to the increased energy supply. When diet was changed post-weaning to be lower in calories, the offspring may have lost fat mass as simply as a result of the caloric deficit.



Only two studies reported differences in female offspring whole body BMC, both of which differ in findings. Howie et al. found that maternal consumption of a high fat diet resulted in higher whole body BMC when compared to female Wistar rat offspring in adulthood from mothers fed control diet (~5 months of age) [44]. However, C57BL/6 females from mothers fed a high fat diet for 1.5 months plus pregnancy and lactation had lowering of whole body BMC at 3.5 months of age, which was also sustained at 6.5 months of age [152]. Results from the study by Devlin et al. [152] parallel previous literature in terms of the impact of direct high fat diet consumption, resulting in overall lowering of whole body BMC [51]. Additionally, it may be that female offspring from mothers fed a high fat diet in the study by Howie et al. [44] were able to effectively utilize the increased nutrient delivery provided by the high fat diet during gestation and suckling to promote optimal bone growth. It is shown that milk from mothers fed a high fat diet have a high level of protein [18], which is known to be imperative in supporting healthy bones [153]. It is difficult to determine whether maternal consumption of a high fat diet truly has a long-lasting impact on female offspring whole body BMC as the only two studies [44, 152] to investigate this have varying results. It may be important to determine overall whole body BMC in addition to changes in BMC at individual bone sites to determine whether a maternal high fat diet has an impact on whole body bone mineral accretion.

Given the evidence provided above and detailed in Table 1, it is evident that a maternal high fat diet can contribute to long-lasting changes in female offspring body composition [44, 149, 151, 152]. Further investigation of the impact of a maternal high fat diet on female offspring body composition should be considered as there are multiple

inconsistencies in study findings. The particular components that may have contributed to discrepancies in studies will be discussed further in Section 3.4. It is of particular importance to examine whether there are long-lasting changes in female offspring body composition, given the rise in obesity in women of child-bearing age [154] and the increased predisposition of disease development with changes in maternal diet, as witnessed by the offspring of the Dutch winter famine [139, 143].

### **3.2. Effect of maternal high fat diet consumption on bone health in female rodent offspring – bone quantity, quality, strength, and fatty acid composition**

Only two studies to date [58, 152] have investigated the effects of maternal high fat feeding on female offspring bone health. Methods and study design for these studies differ from each other, however the animal model and respective high fat diets used are similar [58, 152]. In the first study by Lanham et al., female C57BL/6 mice were fed a control or high fat diet (22% fat by weight – 18% lard, 4% soya oil) for 2.5 months, after which females were mated. Female mice continued to be fed the same respective diets throughout pregnancy and lactation [58]. It is important to note that the high fat diet had additional vitamins and minerals and protein added [58], to account for the greater content of fat, per energy basis. Female offspring from dams fed a control diet were weaned on the control or high fat diet, while offspring from dams fed a high fat diet also continued the high fat diet post-weaning until 7.5 months of age [58]. Femur density and structure were determined using  $\mu$ CT, and femur midpoint peak load measured by biomechanical testing (three-point bending).

This study is the only one to measure the effect of a maternal high fat diet on female offspring bone density. Lanham et al. found that femurs of females from mothers fed control diet and who were fed the high fat diet post-weaning had higher femoral density than females fed control diet post-weaning, from the same mothers. There were no differences in BMD between offspring fed a control diet, from mothers fed the same diet, and offspring fed a high fat diet from mothers fed the same diet [58]. However, femurs of female offspring fed a high fat diet post-weaning and born to control mothers, had the greatest trabecular spacing, which was significantly different from offspring exposed to control or high fat diets both in utero and post-weaning. There were no additional differences in bone volume fraction (bone volume/total volume ratio), trabecular thickness and number of trabeculae (per mm) among female offspring [58]. Despite these alterations in femoral trabecular structure, there were no differences in female offspring femur strength due to a maternal high fat diet [58]. Therefore, it is post-weaning high fat diet consumption is more potent in modulating female offspring bone health in adulthood, compared to maternal high fat diet consumption. Femurs of female offspring fed a high fat diet post-weaning that were born to mothers fed control diet, may have been more susceptible to alterations in density and structure compared to femurs of females fed a high fat diet post-weaning from mothers fed the same diet as there was a mismatch in energy consumption from suckling to post-weaning. Increased femoral BMD in these offspring may be a result of increased energy consumption as there was a greater level of nutrients available to support bone growth. Additionally, the energy level of protein, vitamins, and minerals were increased in the high fat diet. Therefore, offspring were receiving a greater level of calcium and vitamin D, which has been shown to be

imperative for optimal bone growth and development [155, 156]. However, offspring fed a high fat diet post-weaning born to mothers fed a control diet had increased trabecular spacing compared to offspring fed a post-weaning high fat diet from mothers fed the same diet. It may be that in elevated levels, dietary saturated fat intake post-weaning can be harmful to bone structure, rendering them weaker, which has been shown in previous studies [43, 57]. However, considering that female offspring fed a post-weaning diet from mothers fed a high fat diet did not have these adverse effects in femoral trabecular structure, it may be that offspring adapted to this level of fat in utero and suckling. This adaptation is also known as 'predictive adaptive response', which is developmental plasticity due to environmental factors in early life that prepare for the future environment [157]. However, the alterations in trabecular structure did not contribute to differences in bone strength, indicating that there is no increased potential of fracture risk due to a maternal high fat diet.

The study by Lanham et al. tested bone strength solely in the femur, and despite determining structural differences amongst bone types (trabecular versus cortical), bone strength was only measured at a site of cortical bone (i.e. femur midpoint) [58]. Though clinically relevant, femurs are the load bearing bones in humans, therefore it may be important to understand the impact of a maternal high fat diet on other skeletal sites, such as vertebrae which are common sites of fracture in humans [158]. However, it is difficult to appropriately attribute any effects in offspring as a consequence of maternal high fat diet consumption as none of the dietary interventions isolated maternal high fat diet. For example, offspring from mothers fed a high fat diet were continued after weaning on the same high fat diet, making it difficult to decipher whether effects in offspring are due to

**Table 2.** Summary of the literature regarding the impact of a maternal high fat diet on female offspring bone health in rodents

Species	High fat diet (% fat by weight)	Duration of maternal diet	Age of offspring and diet	Methods	Female offspring bone health	Reference
C57BL/6 mice	<b>22% total fat</b> 18% lard 4% soya oil	~2.5 months, pregnancy + lactation	7.5 months (CON or HF)	<b>Mineral:</b> μCT  <b>Structure:</b> μCT  <b>Strength:</b> Biomechanical testing	<b>Mineral (femurs):</b> ↑ Density  <b>Structure (femurs):</b> ↑ CSA Trabecular spacing/number  <b>Strength</b> <b>(femur midpoint):</b> — Peak load	Lanham et al.[58]
C57BL/6 mice	<b>22.5% total fat</b> 19.5% lard 3% soybean oil	1.5 months, pregnancy + lactation	3.5 or 6.5 months (All CON)	<b>Structure:</b> μCT  <b>Strength:</b> Biomechanical testing	<b>Structure</b> <b>(femurs + LV5):</b> — Trabecular spacing/number — Bone area/ trabecular area  <b>Strength</b> <b>(femur midpoint):</b> — Peak load	Devlin et al.[149]

CON – Control diet; HF – High fat diet; μCT – Micro computed tomography; CSA – Cross-sectional area; LV – lumbar vertebra

maternal or post-weaning high fat diet consumption. A dietary intervention that isolates the effects of the maternal high fat diet would have offspring being fed a healthy diet post-weaning. Therefore, although there are persisting effects on female offspring femoral density and structure, it may not be a result of the maternal high fat feeding, as offspring were also fed a high fat diet post-weaning. Therefore, this study may not truly demonstrate nutritional programming in female offspring bone health due to maternal high fat diet consumption.

The second study to examine the impact of maternal high fat diet consumption on female offspring bone structure and strength was conducted by Devlin et al. [152], who studied offspring at two time points, 3.5 and 6.5 months of age. Female C57BL/6 mice were fed a normal or high fat (23% total fat by weight – 20% lard, 3% soybean oil) diet for 6 weeks [152] (age of mice at onset of intervention is unknown). Mice were subsequently bred (12 – 18 weeks of age), and were continued to be fed their respective diets throughout pregnancy and lactation [152]. All offspring at weaning were fed a normal diet until 3.5 or 6.5 months of age. Femoral structure was determined with  $\mu$ CT, and femur midpoint peak load was measured by three-point bending through a materials testing system. At 3.5 months of age, female offspring from mothers fed a high fat diet had greater bone volume fraction (bone volume/total volume) compared to females from mothers fed a normal diet in distal femurs [152]. This effect however was diminished by 6.5 months of age [152], indicating that trabecular bone structure in female offspring from mothers fed a high fat diet is not altered long-term. Additionally, there were no differences in femur midpoint peak load due to maternal high fat diet consumption [152]. Differences in findings from Lanham et al. [58] and Devlin et al.[152] may be attributed

to post-weaning diet, as females from dams fed a high fat diet fed the same diet post-weaning had greater trabecular spacing in femurs at 7.5 months of age in the study by Lanham et al. [58]. Whereas females from dams fed a high fat diet that were fed a control diet post-weaning had no differences in femur structure at 6.5 months of age in the study by Devlin et al. [152]. The finding of no differences in femoral midpoint peak load in this study is in accordance with the findings from Lanham et al., where despite changes in femoral trabecular structure, there were no differences in femur midpoint peak load [58, 152]. However, only cortical bone was utilized for strength measurements. Considering the alterations in trabecular structure, it may be important to determine whether trabecular bone strength is impacted as a result. Like the study by Lanham et al. [58], it is evident that maternal high fat diet consumption does not alter female offspring femoral structure or strength, indicating no programming effect.

Although there are only two studies that have investigated the impact of a maternal high fat diet on female offspring bone health, the findings from these studies appear to be in accordance. Both studies [58, 152] used the same mouse model (C57BL/6), used similar high fat diets, methods for determining bone health, and studied offspring at similar ages. However the study by Lanham et al. [58] increased the level of protein, vitamins, and minerals to account for greater energy content in the high fat diet from the increased level of fat, which was not adjusted for in the study by Devlin et al. [152]. Even despite the lack of adjustment for protein, vitamins, and minerals, in the study by Devlin et al., any effect present at 3.5 months of age did not persist to 7.5 months [152], indicating that a maternal high fat diet did not contribute to long-lasting changes in female offspring bone health. Furthermore, the study by Lanham et al.

demonstrate that a post-weaning high fat diet consumption is more potent in modulating female offspring bone health than a maternal high fat diet *with* post-weaning high fat diet consumption. This may be because offspring have become accustomed to the greater nutrient availability, and have adjusted their physiological responses accordingly. Further research in this particular area is needed, as there remain gaps in the current literature. Studies should consider increasing the levels of protein, vitamins, and minerals in the high fat diet, and have appropriate dietary interventions that allow for more suitable conclusions on whether maternal high fat diet consumption is the main contributor to observed effects in female offspring bone health. Additional research is required to determine whether trabecular bone strength is impacted due to maternal high fat diet consumption, as trabecular structure appears to be altered as a result [152]. No study to date has investigated whether maternal high fat diet consumption alters female offspring bone fatty acid composition. Though it is established that bones are particularly sensitive to dietary fatty acids as discussed previously [63, 73, 74], it is not known whether fatty acid composition changes when high fat diet exposure occurs solely in the previous generation. This information, in addition to measurements of bone mineral, quantity, and strength, will provide a more extensive profile on overall bone health. Furthermore, the role of changing fatty acids in bone and its functional effects on strength require greater characterization. This may be functionally important to determine relative fracture risk or predisposition of developing osteoporosis later in life, as a consequence of early life exposure to maternal over-nutrition.



### **3.3. Effect of maternal high fat diet consumption on female offspring plasma hormone concentrations in rodents**

Hormone levels are often reflective of overall health status. Individuals with a disease will have similar hormonal profiles that reveal underlying health problems. For instance, individuals with metabolic syndrome are typically obese [159], and will have elevated levels of pro-inflammatory cytokines [159-161]. This is reflective of the level of adiposity, which is responsible for the secretion of these cytokines [90, 96]. It is important to study hormones in female offspring to determine whether their hormone profiles are indicative of underlying diseases.

#### **3.3.1. Leptin**

Few studies in animal models have investigated whether a maternal high fat diet can alter circulating levels of leptin in female offspring. The majority of these studies have indicated no differences in leptin concentrations due to maternal high fat feeding [17, 44, 151, 162, 163], all of which have varied duration of exposure to maternal diet, as well as type, and quantity of fat used in maternal diet. Some studies have focused on whether maternal diet during pregnancy-only modulates offspring phenotype, or whether post-natal diet is a contributor to this. Female offspring rats from dams fed a high fat diet throughout pregnancy *and* lactation had higher leptin levels than offspring from dams fed a high fat diet *solely* in pregnancy. This is a clear indication that a post-natal high fat diet exposure from dam's milk during lactation can indeed alter female rat offspring leptin levels in early life (PND10, PND12, PND21) [17, 44, 163]. Howie et al. also found that elevated leptin levels in females from dams fed a high fat diet (for 3.5 months plus

pregnancy and lactation; *or* solely during pregnancy and lactation) are sustained into adulthood (5.5 – 6 months of age) even with a control diet post-weaning. Furthermore, leptin levels were also positively correlated with fat mass in female offspring [44], which corresponds to the literature regarding leptin and adiposity [80].

Two studies in particular contradict the findings of Howie et al. [44]. Connor et al. used the same model (Wistar rat) and high fat diet (24% fat by weight – 21% lard, 3% soybean oil) as Howie et al. [44]. High fat diet exposure occurred solely during pregnancy and lactation, and female offspring from dams fed the high fat diet had no differences in fasted plasma leptin at 4 months of age [151]. It is not clear why differences in results occur in this study by Connor et al. [151] compared to the study by Howie et al. [44], considering both studies had the same experimental model, dietary intervention and method of determining plasma leptin concentrations in female offspring. Differences in results may be attributed to the lack of increased fat mass in female offspring from dams fed a high fat diet in the study by Connor et al. [151], which was a factor in female offspring in the study by Howie et al. [44].

A study by Férézou-Viala et al., [162] also contradicted the findings from Howie et al. [44]. Wistar rat dams were fed a high fat diet (40% total fat by weight – 38% palm oil, 2 % colza oil) for 6 weeks plus pregnancy and lactation, found that control-fed female offspring (~2 months old) from dams fed a high fat diet had no differences in fasting plasma leptin concentrations. Discrepancies from the study by Howie et al., [44] may be due to the duration of maternal high fat feeding, and not necessarily the amount of fat used in the dietary intervention. After reviewing the current literature, a definitive conclusion remains to be established regarding the impact of a maternal high fat diet on

female offspring leptin concentrations. Future investigations in this area of research should consider discovering the specific quantity of fat needed and the appropriate duration of maternal exposure to a high fat diet. This will help better understand the precise situations in which female offspring are predisposed to higher leptin levels, and subsequent risk of disease development in later life.

### 3.3.2. Tumour Necrosis Factor-Alpha

A study by Pantaleão et al. is the only to examine the impact of a maternal high fat diet on female offspring TNF- $\alpha$  levels [163]. Wistar rat dams were fed a high fat diet (40% fat by weight – 33% lard, 7% soybean oil) or control diet for 10 days prior to conception, as well as during pregnancy. Dams were fed either control or high fat diet after parturition throughout lactation. There were no differences in serum TNF- $\alpha$  concentrations in weanling female offspring (3 weeks old) from either dietary intervention (i.e. maternal high fat or control diet; post-weaning high fat or control diet) [163]. This study further provides evidence that a maternal high fat diet during pregnancy may not elicit changes in inflammation measured by TNF- $\alpha$  in female offspring at weaning. A lack of effect by the maternal high fat diet may be due to exposure time of the diet as dams assigned the high fat diet only began consumption days before conception, and were either kept on the same diet or were switched to a control diet at parturition [163]. The duration of maternal high fat diet in this study may not have been adequate to elicit programming in dams, which may have further exacerbated the effects of the high fat diet on female offspring TNF- $\alpha$  concentrations. Perhaps high fat diet consumption at different stages of pregnancy (e.g. early, mid, late gestation) would elicit

programming various effects in offspring. This hypothesis was observed in offspring from mothers exposed to the Dutch winter famine, whereby offspring exposed to famine in early gestation had greater risk of disease development than offspring exposed to famine in mid and late gestation [143]. Further studies are required to appropriately establish the conditions in which a maternal high fat diet can alter female offspring TNF- $\alpha$  concentrations. This will help determine the level of systemic inflammation in female offspring as a result, and disease risk as a consequence.

### 3.3.3. Interleukin-6

The effects of a maternal high fat diet on female offspring IL-6 levels are currently not well characterized. The few studies that have measured IL-6 levels in female offspring have examined mRNA expression in tissues such as the brain [164] and liver [165] as indicators of inflammation or dysfunction of the specific tissue. One study in humans examined plasma IL-6 concentrations in offspring, where offspring born to mothers who gained more weight during pregnancy than recommended had higher IL-6 concentrations than offspring from mothers that gained weight within the recommended range. Gestational weight gain in this study [166] was not measured as a result of high fat diet consumption, however if pregnant women consumed foods higher in dietary fat, an elevated weight may be achieved which could correlate to higher IL-6 levels, however this is an indirect speculation [166]. It is important to measure levels of systemic inflammation in offspring in response to a poor maternal diet to determine whether offspring are predisposed to associated health complications later in life.

#### 3.3.4. MCP-1

There are currently no studies to date that have examined the impact of a maternal high fat diet on female offspring MCP-1 concentrations. This is an important area of discovery as MCP-1 is a known promoter of chronic inflammation commonly observed in obesity [105]. Determining whether offspring from mother's fed a high fat diet have higher levels of MCP-1 throughout life may be beneficial for understanding the offspring's risk of other chronic illnesses potentially as a result of a poor maternal diet. This is especially important with the increase prevalence of obesity in women of child-bearing age [154], and greater consumption of saturated fats due to the Western diet [33].

#### 3.3.5. 17 $\beta$ -Estradiol

Little is known on the effects of a maternal diet high in saturated fat on female offspring estradiol levels. This may be important to determine as estradiol is imperative for proper bone formation [111]. Furthermore, higher levels of estradiol due to maternal diet can predispose female offspring to breast cancer development [167]. This was demonstrated in studies which fed mothers a diet high in *n*6 polyunsaturated fats, which are known to raise estradiol levels [167-169]. It is important to measure levels of estradiol in female offspring to determine whether a maternal high saturated fat diet can permanently alter levels of estradiol and relative risk of disease later in life.

#### 3.3.6. Progesterone

Two studies have directly measured progesterone levels in female offspring born to mothers fed a high saturated fat diet, all of which are in animals models. Both studies

used a similar design, with the same model and high fat diet intervention [151, 170]. Female Wistar rats were fed a high fat (21% lard, 3% soybean oil by weight) or control diet for life (from weaning until ~2.5-3 months), as well as throughout pregnancy and lactation [151], or solely during pregnancy and lactation [170]. There were no differences in plasma progesterone concentrations in female offspring at ~4 and 5 months of age due to life-long maternal high fat feeding [170]. However, these two studies have conflicting results in female offspring progesterone concentrations when maternal high fat diet exposure is restricted to pregnancy and lactation. Sloboda et al. show that ~5 months old female offspring from dams fed a high fat diet, fed control diet post-weaning, had higher progesterone levels than control-fed offspring from dams fed a control diet (throughout pregnancy and lactation). A post-weaning high fat diet did not contribute to any additional alterations in female offspring plasma progesterone concentration [170].

Conversely, Connor et al. show that a maternal high fat diet occurring throughout pregnancy and lactation did not alter female offspring plasma progesterone concentrations at ~4 months of age, regardless of post-weaning diet (i.e. control or high fat) [151]. It is unclear why these two very similar studies have conflicting results. However, with the lack of studies in this area of research, it is difficult to provide a definitive conclusion regarding the influence of a maternal high fat diet on female offspring progesterone concentrations. It is unknown whether diets that contain a greater or lesser amount of fat will have a similar impact on female offspring progesterone concentrations. This may be an important area of future research as progesterone is a known modulator of fat mass distribution and bone health in females [126], and changes

in levels of progesterone may lead to fat mass accumulation in areas associated with disease development [113], as well as potential dysfunction in bone remodelling.

### **3.4. Knowledge gaps**

The literature in previous sections describes the impact of maternal high fat diet consumption on female offspring body composition, bone health, and hormone concentrations. The discrepancies in study findings are difficult to appropriately compare and contrast as there are inherent differences in experimental designs of most studies. Some of the key differences that may have impacted the inconclusiveness of study findings are: experimental models, quantity of fat supplied in the high fat diet, age of offspring studied, and level of protein and micronutrients in the high fat diet. Detailed discussion of the key differences among studies are the following:

**i. Differences in the experimental model used amongst studies.** Although all of the studies described above used rodents, there were differences in strains used. More specifically, three different strains of rodents were used amongst studies: Wistar rats, Sprague Dawley rats, and C57BL/6 mice. In regards to the literature, rats were more resistant to changes in body composition compared to C57BL/6 mice. These mice had evident changes in body composition (i.e. increased fat mass, lower lean mass) due to maternal consumption of a high fat diet [149, 150]. However, rats with similar changes in body composition had also consumed a post-weaning high fat diet [17, 151], which may have exacerbated the sole effects of the maternal high fat diet. Though the literature regarding programming of bone health has only been investigated in C57BL/6 mice, it

may be important to determine whether these same effects are evident in other species, specifically rats, as these are the preferred model for examining osteoporosis and bone health [171].

Although these rodents are the preferred models when using a high fat diet (due to the responsiveness to diet-induced obesity [48]), there may be specific strain differences that could impact the sensitivity of the rodent to the high fat diet, or relative resistance to the development of health complications as a result. Furthermore, changes in growth rate between species may also differ (i.e. mice versus rats), which could impact overall metabolism and rate of bone mass accretion.

**ii. The quantity of fat used in the dietary interventions is inconsistent among studies.**

The quantity of fat amongst studies discussed in the literature has greatly varied. Some studies reported using a diet containing as high as 40% fat by weight [17, 150], while others used a diet containing 22% fat by weight [149, 152]. Generally, studies in which offspring were fed a healthy diet post-weaning found similar alterations in female offspring body composition (i.e. increases in fat mass, lower lean mass) [44, 149, 150]. However the minimum quantity of fat required in the maternal diet that is required to elicit these changes in female offspring body composition occur is unknown. A higher level of fat in the maternal high fat diet) may have induced further changes in female offspring compared to studies using a lower level of fat. For example, a diet that has a higher quantity of fat (40% fat by weight [17, 150]) may induce obesity in mothers, which may therefore predispose offspring to become obese as well [172], compared to offspring from mothers fed a diet with a lower fat content (22% fat by weight [149, 152]).



However, the two studies that implemented a diet with 40% fat by weight studied offspring at a young age (day of birth [150] and weaning [17]), therefore it is difficult to compare findings to other studies that used lower quantities of fat and examined offspring in adulthood.

Although the two studies that investigated female offspring bone health had diets containing a similar level of fat in the maternal diet, there are differences in results. Offspring in the study by Lanham et al. [173] had greater femoral density, and increased trabecular spacing. However, Devlin et al. [152] found no differences in offspring bone health due to maternal consumption of a high fat diet. Despite similar quantities of fat in the experimental diets, it is difficult to appropriately compare and contrast these study findings as offspring were fed different post-weaning diets. The quantity of fat in the high fat diet may dictate the severity of health complications in offspring, and whether these complications are truly long-lasting, indicative of programming.

**iii. The age at which offspring were studied differed among studies.** Most studies have examined female offspring during adulthood [44, 149, 151, 152, 173], a time in which growth is slow. It may be particularly important to highlight changes in female offspring body composition and bone health at different stages of life, particularly in periods of rapid growth and development (i.e. early life), and stages of slower growth (i.e. adulthood). This can help decipher whether any changes that exist in early life truly remain persistent in adulthood, which would suggest programming.

**iv. Post-natal or post-weaning diet consumption of offspring.** In reviewing the literature, it is evident that offspring fed a post-natal or post-weaning high fat diet had greater changes in body composition (i.e. increased fat mass, lower lean mass) [17] than those fed a healthier diet [44, 151, 152]. Furthermore, female offspring that consumed a high fat diet post-weaning had greater changes in bone health (i.e. increased femoral density, cross sectional area, trabecular spacing) [173], whereas offspring fed a healthy diet found no changes in bone due to maternal high fat diet consumption [152]. In order to appropriately attribute any changes in offspring body composition to maternal consumption of a high fat diet, it is important for offspring to consume a healthy diet postnatally and/or post-weaning. If offspring are continued on the same high fat diet as their mother's throughout life, it would be impossible to determine whether any differences in body composition and/or bone health were due to exposure of the maternal diet or the post-weaning diet.

**v. Level of protein, vitamins and minerals in the high fat diet.** Only one study that examined female offspring body composition (Elahi et al. [149]) and only one study that examined female offspring bone health (Lanham et al. [58]) in the literature described previously increased the level of proteins, vitamins, and minerals in the maternal high fat diet. It is important to increase the level of protein, vitamins, and minerals in the high fat diet as overall energy has increased. It is well documented that calcium and vitamin D are imperative for optimal bone growth [174, 175] and maintenance [176, 177]. Studies also show importance for adequate protein intake as low dietary protein consumption in rats causes impaired bone formation [178], and increases risk of fracture in the elderly [179,

180]. The elevation of these important nutrients would ensure that appropriate levels of each nutrient would be maintained, per energy basis. This is to ensure that any persistent changes evident in female offspring body composition and/or bone health could appropriately be attributed to the level of fat in the diet, rather than insufficient intake of these nutrients.

After reviewing the literature and the apparent inconsistencies amongst studies, it is important for research to continue in the area of maternal high fat diet consumption and its impact on female offspring body composition and bone health. This research would help bridge gaps in current discrepancies in findings from the literature.

#### **Chapter 4.0: Objectives and Hypotheses**

The *overall objective* of this work was to determine if long-term maternal high saturated fat (HSF) feeding, as well as throughout pregnancy and lactation, had adverse effects on body composition and bone health in female offspring in a Wistar rat model. The Wistar rat is an established model for high fat feeding, as this model develops chronic diseases (obesity, glucose intolerance) similar to how humans react upon long-term consumption of a high fat diet [48]. This rat model is also an established model for the study of osteoporosis and its prevention [171]. The rat is a suitable experimental model to examine human bone growth and development patterns are similar. Particularly, after rapid growth in rats, where peak bone mass is attained at 10 months of age [181, 182], bone remodelling is favoured over bone modelling, as occurs in humans [183]. Plasma hormones were measured as subsequent markers of metabolic status and additional contributors to alterations in body composition and/or bone homeostasis.

Specific objectives were to determine the effect of maternal exposure to a HSF diet on female offspring for the following outcomes:

- i. *Body composition* – lean, fat and bone mass, as well as fat mass distribution; and
- ii. *Bone health* – specifically bone quantity (BMC, BMD) and strength (peak load) in femurs and lumbar vertebrae, and femur fatty acid composition.

Each of the above mentioned objectives were examined in female offspring only. Knowing that programming effects may be evident over generations due to epigenetic modifications, it is important to study effects in female offspring. This is because any effect that occurs in female offspring may transfer to their offspring [139, 145-147]. All female offspring were studied at two time points: *weaning* and *three months*. It is important to study offspring in early life (weaning) and young adulthood (3 months of age) to determine whether any apparent alterations in body composition or bone health at weaning (when offspring are still suckling) persist into adult life; a persistent effect would be indicative of programming due to maternal diet.

In accordance with the specific objectives, we *hypothesized* that female offspring of dams fed a HSF fat diet at weaning and three months of age would have:

- *Lower* lean and bone mass, with *higher* fat mass
- *Greater* fat accumulation in visceral fat pads compared to subcutaneous fat pads, indicating metabolic distress
- *Lower* BMC and BMD in both femurs and lumbar vertebrae
- *Higher* saturated fatty acid content in femurs

- Lower peak load in femurs and lumbar vertebrae

## **Chapter 5.0: Study Design**

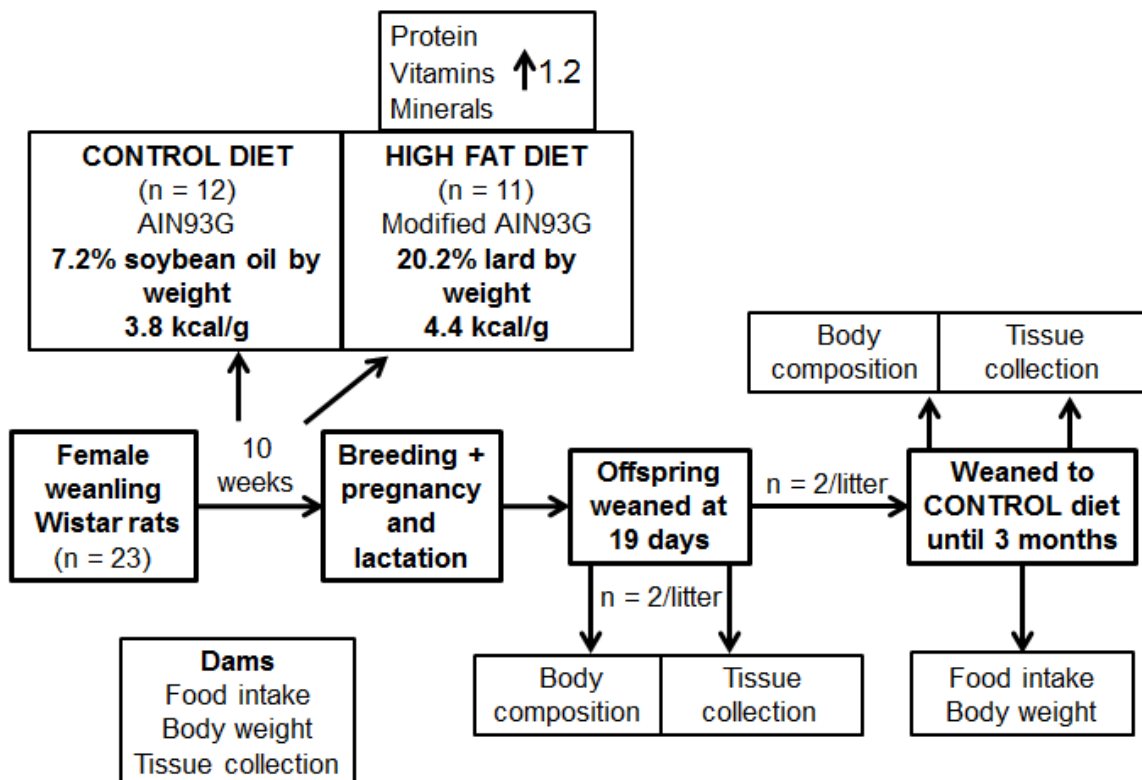
All experimental procedures were approved by the Brock University Animal Care Committee, and complied with the Canadian Council on Animal Care [184]. Female Wistar rats (n = 23) were obtained at weaning (~28 days old) and housed two per cage with a 12:12 hour light/dark cycle. Rats were housed two per cage for environmental enrichment, and to ensure rats had reduced anxiety and neural deficits, as rats in isolation often demonstrate [185]. Rats were randomly assigned to receive one of two diets; a control (AIN93G diet, 7% soybean oil by weight) or HSF diet (modified AIN93G diet, 20% lard by weight) *ad libitum* for 10 weeks starting at 1 week post-weaning (28 days of age). The CON diet contained 18.8% protein, 63.9% carbohydrate, and 17.2% energy from fat (TD.94045; Harlan Teklad, Mississauga, ON). The HSF diet contained 19.1% protein, 39.9 % carbohydrate, and 41.0% energy from fat (TD.02016; Harlan Teklad). Both the type and quantity of dietary fat were altered in the HSF diet compared to CON diet. This was done to best represent the typical dietary fat consumption as observed in the modern Canadian diet, which is associated with elevated intake of foods rich in saturated fat (i.e. red meat) [186]. The amount of protein, vitamins, and minerals in the HSF diet were increased by a factor of 1.2 compared to the control diet to match the increased energy content (3.8 kcal/g CON diet *versus* 4.4 kcal/g HSF diet) (Table 3). This was done to ensure that any observed effects in offspring can be attributed to the higher level of fat in the diet, and not due to a lower relative energy intake of these nutrients. As the level of fat increases, the relative energy contribution of proteins and micronutrients

is lowered as a result. Therefore, the level of protein and micronutrients were increased by the same factor per kg of diet as the dietary fat to ensure the relative contribution of these nutrients was similar to that of a healthy diet. Food intake was measured three times a week using an electronic scale (VI-1200, Acculab, Bradford, MA, USA). Food intake was determined by weighing, recording, and subtracting the amount of food remaining by the new food provided in one cage for that day. To account for the number of rats in a cage, the total food intake per cage was divided by 2 (2 rats per cage). Although pair feeding would appropriately control for energy intake amongst dietary conditions, individuals that habitually consume a HSF diet may also consume above their daily caloric requirements. Therefore to best represent the dietary pattern of the Canadian diet (where 50% of women and 70% of men consume above their daily caloric requirements [187]), energy consumption amongst dietary groups was not controlled for. Body weights were recorded weekly using an electronic scale (VI-1200, Acculab).

After 10 weeks of feeding, females were bred with male Wistar rats ( $n = 6$ ) of similar age. Each male rat was bred with the same number of females from each intervention to ensure equal paternal influence among groups. Males were placed in a cage of female rats until successful mating ( $\sim 1$  week), which was established in females by an increase in body weight greater than normal weekly weight gain (as determined by female Wistar rat growth chart). After pregnancy confirmation, female rats were continued on their assigned diets throughout pregnancy and lactation. Offspring were weaned at PND 19. Dams were fasted overnight and euthanized the following morning by an overdose of sodium pentobarbital (12 mg/100 g body weight). Blood, bones (femurs and lumbar vertebrae), and fat pads (retroperitoneal, omental, gonadal) were

collected. Two male and 2 female offspring per litter were euthanized at weaning – 1 male and 1 female were used for body composition measurements, while the other male and female from the litter were used for tissue collection (blood, bones, organs, fat pads).

Additionally, 2 males and 2 females per litter were weaned onto CON diet and studied until 3 months of age. Offspring weaned to CON diet were selected by body weight; where 2 female and 2 male offspring with the median body weights (in the range of body weights of all female or all male offspring per litter) were selected. Food intake was measured 3 times per week, while body weight was recorded once weekly from weaning to 3 months of age. To ensure no “litter effect” (“litter effect” refers to the fact that siblings will have more similar characteristics than offspring who are not siblings,



**Figure 2.** Flow chart depiction of the study design

and thus including more than one offspring per sex per litter may influence findings by over-representation of a specific characteristic) [188], only 1 male and 1 female per litter were reported for each outcome.

**Table 3.** Dietary composition of experimental diets

<b>Ingredient</b>	<b>CON</b>	<b>HSF</b>
Soybean oil (g/kg)	70	-
Lard (g/kg)	-	200
Casein (g/kg)	200	240
L-Cysteine (g/kg)	3	3.6
Corn starch (g/kg)	397.49	217.38
Maltodextrin (g/kg)	132	132
Sucrose (g/kg)	100	100
Cellulose (g/kg)	50	50
Vitamin mix (AIN93G-VX) (g/kg)	10	12
Mineral mix (AIN93G-MX) (g/kg)	35	42
<i>Quantity of vitamins and minerals</i>		
Calcium (g/kg)	5.04	6.05
Phosphorus (g/kg)	2.96	3.55
Magnesium (g/kg)	0.51	0.62
Folic acid (mg/kg)	2	2.4
Vitamin A (IU/kg)	4000	4800
Vitamin D (IU/kg)	1000	1200
Vitamin E (IU/kg)	75	90
Vitamin K (mg/kg)	0.75	0.9
<i>Energy contribution of nutrient (% kcal)</i>		
Fat	17.2	41
Protein	18.8	19.1
Carbohydrate	63.9	39.8
<i>Relative quantity of nutrients (% by weight)</i>		
Fat	7.2	20.2
Protein	17.7	21.2
Carbohydrate	60.1	44.2

CON – AIN93G (TD.94045; Harlan Teklad); HSF – modified AIN93G (TD.02016; Harlan Teklad)



**Table 4.** Fatty acid composition of experimental diets

Fatty acid	CON	HSF
14:0	0.59 ± 0.01	2.31 ± 0.03
16:0	12.25 ± 0.10	25.79 ± 0.03
18:0	4.19 ± 0.02	11.95 ± 0.21
16:1	0.20 ± 0.01	2.75 ± 0.01
18:1	20.42 ± 0.36	36.34 ± 0.17
18:3n3	8.46 ± 0.06	0.82 ± 0.01
18:2n6	51.91 ± 0.31	16.38 ± 0.29
Total SFAs	18.52 ± 0.10	41.67 ± 0.12
Total MUFAs	21.01 ± 0.36	40.10 ± 0.21
n3 PUFAs	8.46 ± 0.06	1.10 ± 0.01
n6 PUFAs	52.00 ± 0.30	17.14 ± 0.29

Values are expressed as percent mole fraction of total fatty acids, percent mole fraction of fatty acids below 1% for both diets are not shown; CON, AIN93G diet; HSF, AIN93G with 20% lard by weight; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

At 3 months of age, similar to weaning, 2 male and 2 female offspring per litter were euthanized with an overdose of sodium pentobarbital after an overnight fast. One rat per gender was used for body composition measurements while one rat per gender was used for tissue collection (same as dams and weanling offspring). All blood samples, excised bones, and half of the sampled fat pads (per rat) were stored at -80°C until analyses. The other half of sampled fat pads were stored in scintillation vials containing 10% formalin (buffered neutral), and archived. It is important to note that subsequent outcomes in this study were conducted solely in female offspring; male offspring were examined in a separate study.

## **Chapter 6.0: Methodology**

### **6.1. Distribution of adipose tissue**

The distribution of adipose tissue was determined by individual extraction and weighing of fat pads. Fat pads were sampled at 3 different sites: omental, retroperitoneal, and parametrial. Fat pads were carefully excised in dams and female offspring (weaning and 3 months of age) after euthanasia, and weighed on an electronic scale (VI-1200, Acculab). Individual, as well as the sum of all fat pads collected, were compared. Fat pad comparisons between groups examined differences in absolute and relative weights. Relative weights were calculated by adjusting for body weight. Relative fat pad masses were calculated as follows: *fat pad mass (g) / body mass (kg)*.

## **6.2. Body composition assessment**

Body composition (lean, fat and bone mass) was determined using DXA (pSabre, Orthometrix, Naples, FL, USA) and specialized software (Host Software version 3.9.4; Scanner Software version 1.2.0) at weaning and 3 months of age. All rats were anaesthetized with isoflurane, and placed on the scanning field in a prone position (nose facing left side of scanning field) prior to analysis. In weanling females, the region of interest (ROI) surrounded the abdominal region (4 cm in width, 3.5 cm in height), with the lower boundary immediately above the femurs heads [189] and scan parameters of 10 mm/s (speed) and a resolution of 0.2 x 0.2 mm. Three month old offspring were scanned with the ROI 10 cm in width and 7 cm in height, fixed in the abdominal region, again with the lower boundary immediately above the femur heads [189] and scan parameters of 10 mm/s (speed) with a resolution of 0.5 x 1.0 mm. Percent lean, fat, and bone mass were calculated by dividing lean, fat, or bone mass in grams by the total mass of the ROI

and multiplying by 100. Coefficients of variation for weanling female offspring % bone mass, % lean mass, and % fat mass were as follows: 6.7%, 1.8%, and 9.7%.

### **6.3. Collection of plasma and hormone analysis**

Blood was collected in EDTA tubes and centrifuged at 3000 RPM for 10 minutes at 4°C to obtain plasma from dams and female offspring (weaning and 3 months of age). Multiplex immunoassays for metabolic hormones were completed using Magpix technology with xPONENT software (Luminex Corporation, Austin, TX, USA). Plasma from dams and all female offspring were measured for leptin, TNF- $\alpha$ , IL-6, and MCP-1 using a Rat Metabolic Magnetic Bead Panel (Cat. #RMHMAG-84K; Millipore Corporation, Billerica, MA, USA). Plasma from dams and 3 month old females were measured for 17 $\beta$ -estradiol and progesterone using a Steroid/Thyroid Hormone Magnetic Bead Panel (Cat.#STTHMAG-21K; Millipore Corporation). Samples were pipetted (25  $\mu$ L) in a 96-well plate in triplicate along with quality controls and standards in buffer. The plate was incubated (in the dark) with antibody immobilized beads at 4°C on a shaker (low speed) overnight. After washing samples, detection antibodies (50  $\mu$ L) were added and incubated on shaker (30 minutes in the dark), followed by another incubation (30 minutes in the dark) with the addition of Streptavidin-Phycoerythrin (50  $\mu$ L) before running the plate.

Unlike the Metabolic Magnetic Bead Panel, samples for the Steroid/Thyroid Hormone Magnetic Bead Panel were prepped with acetonitrile precipitation. Acetonitrile (375  $\mu$ L) was added to samples, vortexed (~5 seconds) and left to settle for 10 minutes. Samples were vortexed again (~5 seconds), and subsequently centrifuged for 5 minutes at

17000 g. Five hundred microliters of supernatant was removed from each sample, and dried using a refrigerated Speed Vac (Refrigerated CentriVap Concentrator, Labconco, Kansas City, MO, USA) on the highest vacuum setting. Dried samples were reconstituted in buffer (200  $\mu$ L) and placed on a shaker for 10 minutes. The immunoassay protocol is similar to the Metabolic panel kit described above, with the addition of horseradish peroxidase conjugate (25  $\mu$ L) to each of the wells before the addition of antibody immobilized beads, with an overnight incubation on a shaker at 4°C (in the dark) following. Twenty-five microliters of detection antibody was added to the plate, sealed, covered and agitated (low speed) for 1 hour. Twenty-five microliters of Streptavidin-Phycoerythrin was added to each well, agitated (low speed) for 30 minutes, and subsequently washed. The plate was run with buffer using the same software as described above.

#### **6.4. Bone quantity of excised femurs and LV1-3**

Bone quantity of femurs and lumbar vertebrae 1-3 (LV1-3) was measured as BMC (mg) and BMD ( $\text{mg}/\text{mm}^2$ ) in dams and female offspring (weaning and 3 months of age) using DXA (pSabre, Orthometrix) and specialized software (host software version 3.9.4; scanner software version 1.2.0) [63]. Since offspring at weaning are still growing, BMC was adjusted for the length of the femur during analysis. Right femurs were scanned as a whole, and additionally at the 1/3<sup>rd</sup> proximal end as there is more trabecular bone at this site. The 1/3<sup>rd</sup> proximal femur is also the site at which a hip fracture would occur (femur neck) in humans. Femurs were placed on the scan field in the upper right quadrant, with the distal femur at the vertical axis, laid on its posterior surface (femur

head facing the operator). The 1/3<sup>rd</sup> proximal femur was determined by dividing the length of the whole femur by 3, and placing an additional ROI with the calculate length at the proximal end. LV1-3 was also positioned in the upper right quadrant laid on its spinous processes, with LV3 proximal to the vertical axis. The femurs and LV1-3 from weanling offspring were scanned at a speed of 2 mm/sec and a resolution of 0.1 x 0.1 mm. Coefficients of variation for BMC of the whole femur and LV1-3 in weanling females are: 2.4% and 5.2%. Coefficients of variation in weanlings for BMD of the whole femur and LV1-3 are: 4.6% and 1.7%. Femurs and LV1-3 from dams and 3 month female offspring were analyzed at a speed of 10 mm/sec, and a resolution of 0.2 x 0.2 mm. Coefficients of variation in BMC of the whole femur and LV1-3 in 3 month old female offspring were: 0.2% and 1.3%. Coefficients of variation in 3 month old female offspring for BMD of the whole femur and LV1-3 were: 0.3% and 0.6%, respectively.

### **6.5. Biomechanical strength of femurs and LV3**

Bone strength in femurs and LV3 of dams and 3 month old female offspring was determined as peak load, which is the maximum force a bone can withstand before fracturing. Peak load was measured at the femur midpoint, femur neck, and 3<sup>rd</sup> lumbar vertebrae using a materials testing system (Model 4442, Instron, Norwood, MA, USA) and specialized software (Series IX Automated Materials Tester, version 8.1 5.00, Instron). To determine peak load at femur midpoint, the femur was laid on its posterior surface on two metal holders spaced ~14 mm apart. A stainless steel crosshead was lowered perpendicularly to the femur at a constant speed of 2 mm/min, until peak load was achieved (i.e. fracture). Once the femur fractured, the proximal end of the femur was

placed in a customized jig that allowed the femur to be fixed in a vertical position (femur head facing up). The stainless steel crosshead (with a flat, circular surface) was lowered at the same speed, perpendicular to the femur neck until fracture. To determine peak load of LV3, LV3 was fixed upright on a metal custom jig with a circular, flat surface. The crosshead (the same the custom jig) was slowly lowered at 2 mm/min which subsequently compressed the vertebra until fracture [63]. Peak load was quantified (in Newtons) by observing the highest peak from the load-displacement curve generated from software.

## **6.6. Lipid composition in femurs**

Lipid composition of left femurs from dams and female offspring (weaning and 3 months of age) was determined in cortical bone. Femurs were sliced in half using a band saw. Bone marrow was discarded and fragments of bone were wrapped in aluminum foil and placed in liquid nitrogen. The bone fragments were hammered and then pulverized using a mortar and pestle under liquid nitrogen [190]. The resultant powder was added into 15 mL glass screw cap Kimax tubes and total lipids were extracted with 2:1 chloroform:methanol [191]. Extracted lipids were dried under nitrogen, weighed, and reconstituted in 2 mL of chloroform to form the lipid stock. Total lipid from each sample was saponified and methylated [190]. Briefly, 0.0625 mg of lipid from each lipid stock was saponified and methylated at 100 °C using 0.5 M of potassium hydroxide and 6% H<sub>2</sub>SO<sub>4</sub>-MeOH, respectively. The resulting fatty acid methyl esters were separated on a UFM-RTX WAX analytical column (Thermo Electron Corp., Milan, Italy) using gas chromatography (Trace GC Ultra, Thermo Electron Corp.) fitted with a fast flame

ionization detector, a split-splitless injector, and Triplus AS autosampler [30]. Fatty acids were identified by comparison of retention times with those of a known standard (Supelco 37 component FAME mix, Supelco, Bellefonte, PA, USA) and absolute amounts of individual fatty acids were calculated with the aid of an internal standard, tridecanoic acid (13:0), added to the samples prior to methylation.

## **6.7. Statistical analyses**

All statistical analyses were completed using SigmaStat (version 3.5, Systat, Chicago, IL, USA). For dams, independent samples t-tests were used for body weight (at breeding and 6 months of age), fat pad mass (absolute and relative to body weight), plasma hormones (leptin, TNF- $\alpha$ , IL-6, MCP-1, 17 $\beta$ -estradiol, progesterone), bone mineral (femurs and LV1-3), bone strength (femur and LV3), and femur lipid profile. A repeated measures ANOVA followed by a Student-Newman Keul's (SNK) *post-hoc* analysis, was used to determine food intake in dams. For female offspring, a repeated measures 2-way ANOVA, followed by SNK *post-hoc* analysis was used to analyze body weight and food intake, with offspring age and maternal diet used as the two factors. Offspring body composition (lean, fat, and bone mass expressed as % of ROI), fat pad weights (retroperitoneal, omental, parametrial), plasma hormones (leptin, TNF- $\alpha$ , IL-6, MCP-1, 17 $\beta$ -estradiol, progesterone), bone mineral (femurs and LV1-3), bone strength (femur and LV3), and femur lipid profile were analyzed using 2-way ANOVA with SNK *post-hoc* analysis. Independent samples t-tests were conducted for litter comparisons (litter number, male:female ratio, weight), as well as 17 $\beta$ -estradiol and progesterone levels as there was only sufficient quantity of sample for measurement at 3 months of

age. Independent samples t-tests were also used to conduct bone strength in female offspring at 3 months of age as bones at weaning were too small for the strength analyses. Data are expressed as mean  $\pm$  SEM. Statistical significance was determined at  $p \leq 0.05$ .

## **Chapter 7.0: Results**

### **7.1. Litter characteristics**

There were no differences in litter size, or litter weight at PND3. Differences in litter weight were detected at PND12 and weaning (PND19) where offspring from dams fed the HSF diet were heavier than those from dams fed CON diet (Table 5).

**Table 5.** Litter characteristics in offspring from dams fed CON or HSF diet

	<b>Maternal CON</b>	<b>Maternal HSF</b>	<b><i>p</i> Values</b>
<b>Litter size</b>	15 $\pm$ 1	14 $\pm$ 1	0.598
<b><i>Litter weight (g)</i></b>			
<b>Postnatal day 3</b>	133 $\pm$ 4	136 $\pm$ 10	0.797
<b>Postnatal day 12</b>	386 $\pm$ 6	438 $\pm$ 14	<0.001
<b>Weaning weight</b>	52 $\pm$ 2	63 $\pm$ 2	< 0.001

Values are expressed as mean  $\pm$  SEM, n = 15 and n = 14 for offspring from dams fed CON or HSF diet, n = 9 for comparisons of weaning weight.

### **7.2. Effect of high saturated fat feeding in dams**

#### **7.2.1. Pregnancy**

There were no differences in the number of females that became pregnant between diet groups. Nine out of 12 dams fed HSF and 9 out of 11 dams that were fed CON diet became pregnant.

#### **7.2.2. Food intake and body weight of dams**



**Table 6.** Fat pad mass, plasma hormones, and bone mineral and strength in dams

<b>Outcomes</b>	<b>CON</b>	<b>HSF</b>	<b><i>p</i> Value</b>
<b>Body weight (g)</b>	324 ± 5	350 ± 10	0.028
<b>Fat pad mass</b>			
Retroperitoneal (g)	4.93 ± 0.41	6.18 ± 0.60	0.104
(g/kg body weight)	15.24 ± 1.29	17.50 ± 1.34	0.244
Omental (g)	4.30 ± 0.35	5.47 ± 0.45	0.055
(g/kg body weight)	13.51 ± 1.20	15.61 ± 1.08	0.212
Parametrial (g)	6.01 ± 0.48	8.0 ± 0.90	0.068
(g/kg body weight)	18.59 ± 1.50	22.69 ± 2.26	0.150
Total (g)	15.24 ± 1.03	19.65 ± 1.86	0.055
(g/kg body weight)	47.18 ± 3.38	55.80 ± 4.42	0.141
<b>Plasma Hormones</b>			
Leptin (pg/mL)	1357.7 ± 158.5	1936.8 ± 331.8	0.135
TNF-α (pg/mL)	BD	BD	
IL-6 (pg/mL)	227.7 ± 63.1	506.3 ± 122.4	0.130
MCP-1 (pg/mL)	391.2 ± 122.8	475.2 ± 124.9	0.525
17β-estradiol (ng/mL)	0.03 ± 0.01	0.04 ± 0.01	0.485
Progesterone (ng/mL)	21.5 ± 3.4	29.4 ± 3.2	0.110
<b>Bone Mineral</b>			
<b><i>Whole femur</i></b>			
BMC (mg)	355.9 ± 15.4	367.0 ± 13.4	0.584
BMD (mg/mm <sup>2</sup> )	1.70 ± 0.05	1.76 ± 0.04	0.379
<b><i>1/3 Proximal femur</i></b>			
BMC (mg)	122.0 ± 6.2	123.9 ± 4.6	0.800
BMD (mg/mm <sup>2</sup> )	1.62 ± 0.05	1.69 ± 0.05	0.332
<b><i>LV1-3</i></b>			
BMC (mg)	244.5 ± 15.6	249.5 ± 11.5	0.792
BMD (mg/mm <sup>2</sup> )	1.45 ± 0.05	1.48 ± 0.04	0.625
<b>Bone Strength</b>			
<b><i>Femur midpoint</i></b>			
Peak load (N)	125.4 ± 9.1	138.3 ± 7.1	0.948
<b><i>Femur neck</i></b>			
Peak load (N)	82.9 ± 17.4	62.3 ± 8.2	0.300
<b><i>LV3</i></b>			
Peak load (N)	188.2 ± 18.4	220.4 ± 17.2	0.219

Values are expressed as mean ± SEM (n = 9 per group). BD, values below the level of detection, no comparison made.

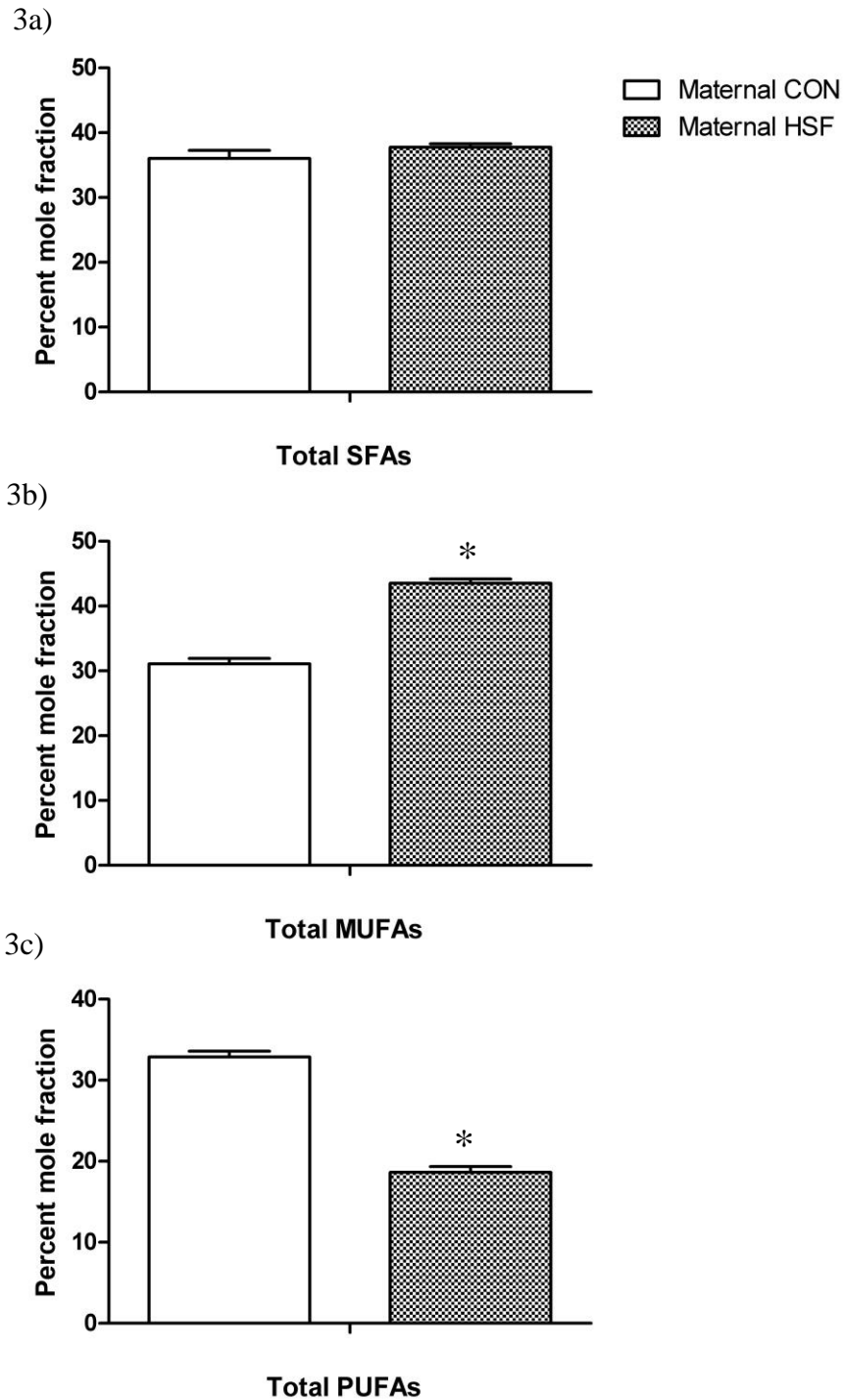
There were no differences in body weight between rats assigned to HSF or CON upon randomization. There was an interaction of maternal diet and time (weeks), while comparing food intake (kcal consumed per rat per day) in dams. Dams fed a HSF diet consumed more energy per day in 8 of 10 weeks ( $p < 0.05$ ) compared to dams fed CON diet, with no differences at week 5 ( $p = 0.637$ ) and week 8 ( $p = 0.096$ ). Food intake was not measured during breeding or pregnancy and lactation, to avoid inconsistencies in food measurement with the presence of a male rat, and to reduce cage disruptions. There were no differences in body weight over 10 weeks of feeding prior to breeding, or at time of breeding. However, dams fed a HSF diet were heavier at necropsy than dams fed CON diet (Table 6).

#### 7.2.3. Fat pad masses of dams

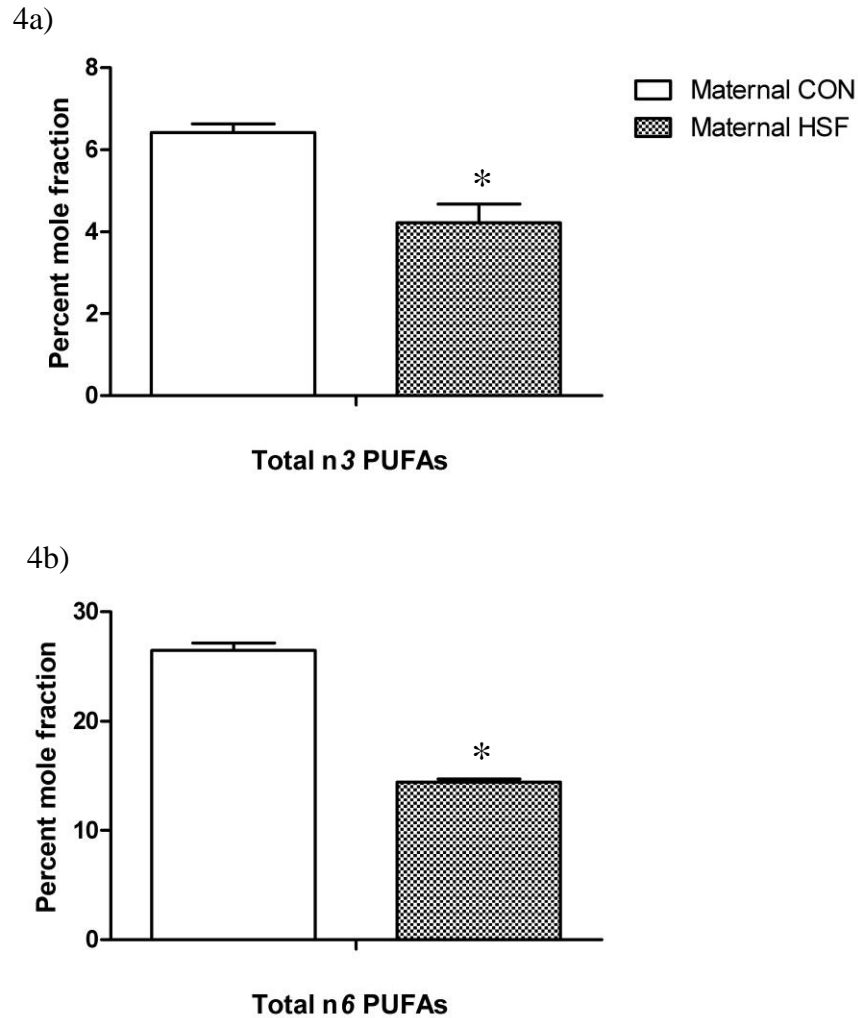
No differences were detected when comparing individual (retroperitoneal, omental, parametrial) and total fat pad weights. There were no additional differences in fat pad mass when adjusted for body weight (Table 6).

#### 7.2.4. Plasma hormones of dams

No differences were detected in plasma concentrations of leptin, IL-6, MCP-1, 17 $\beta$ -estradiol and progesterone between dams fed HSF *versus* CON diet (Table 6). Levels of plasma TNF-  $\alpha$  for some rats were below the minimum level of detection for the assay, therefore no comparison was made for plasma TNF- $\alpha$ . However, there was a greater number of dams fed HSF diet ( $n = 5$ ) with detectable levels of TNF- $\alpha$  compared to dams fed CON diet ( $n = 2$ ) (Table 6).



**Figure 3.** Percent mole fractions of fatty acids in femurs of dams: **a)** total SFAs, **b)** total MUFAs, and **c)** total PUFAs. Values are expressed as mean  $\pm$  SEM.  $n = 7$  or  $8/\text{group}$ . \* Values are significantly different from maternal CON ( $p < 0.05$ ). CON: control diet; HSF: high saturated fat diet; SFAs: saturates; MUFAs: monounsaturates; PUFAs, polyunsaturates.



**Figure 4.** Percent mole fractions of PUFAs in femurs of dams: **a)** total *n*3 PUFAs and **b)** total *n*6 PUFAs. Values are expressed as mean  $\pm$  SEM. *n* = 7 or 8/group. \* Values are significantly different from maternal CON ( $p < 0.05$ ). CON: control diet; HFS: high saturated fat diet; PUFAs: polyunsaturates.

#### 7.2.5. Bone mineral and strength in femurs and lumbar vertebrae of dams

There were no differences in BMC and BMD of the whole femur and the 1/3<sup>rd</sup> proximal femur, as well as no differences in LV1-3 in dams fed HSF versus CON diet. Furthermore, no differences were detected in peak load of the femur midpoint, femur neck, or LV3 (Table 6).

#### 7.2.6. Fatty acid composition of femurs of dams

Dams fed a HSF diet had greater percent mole fractions of total MUFAs and total PUFAs than dams fed CON diet ( $p < 0.001$ ) (Figure 3). The greater percent mole fractions of total PUFAs corresponded with significant increases in  $n3$  and  $n6$  PUFAs ( $p < 0.001$ ) (Figure 4). However there were no differences in total SFA content between dams fed HSF versus CON diet ( $p < 0.001$ ) (Figure 3).

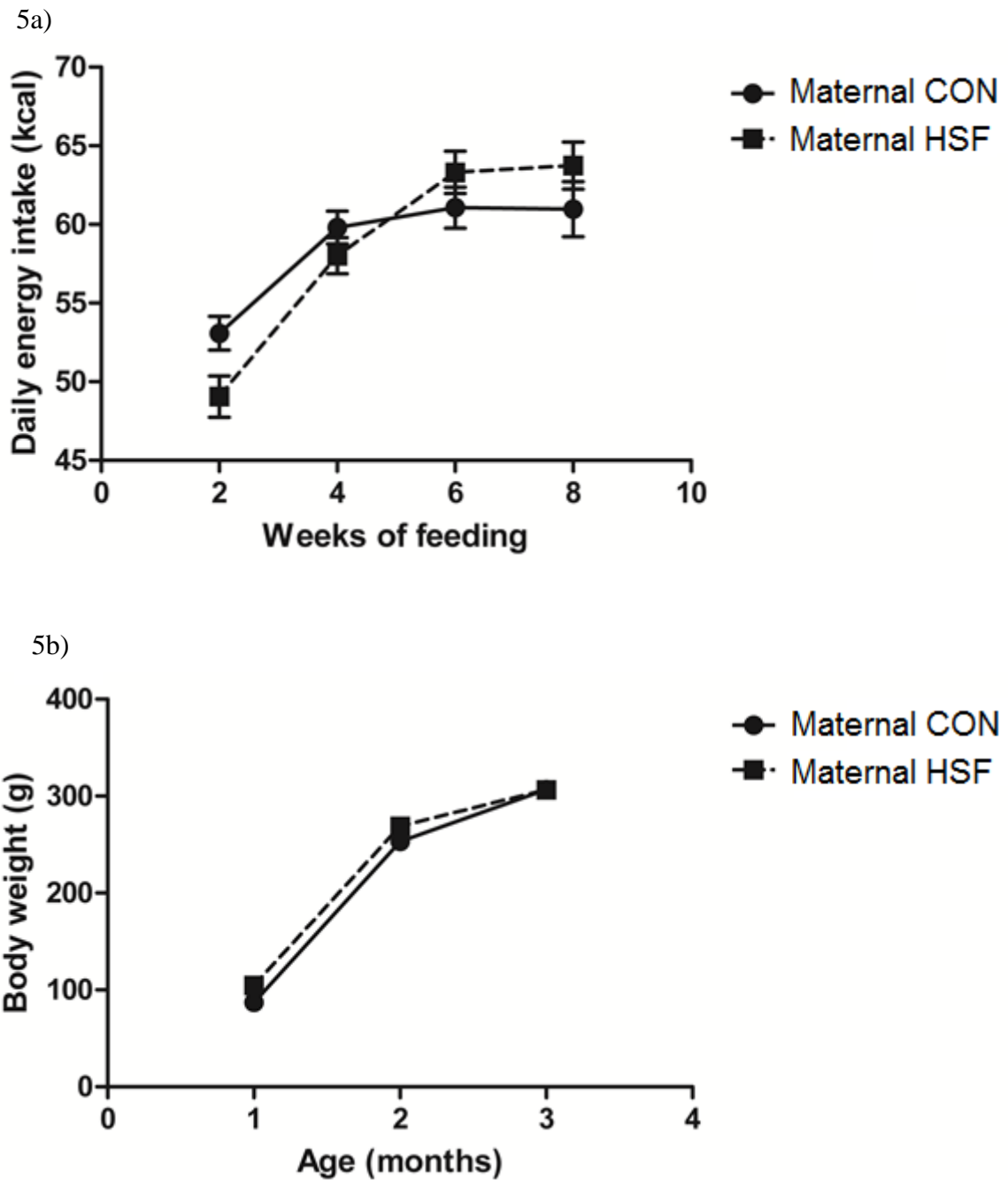
### 7.3. Effect of maternal high fat diet on female offspring

#### 7.3.1. Food intake and body weight of female offspring

There was an interaction between maternal diet and offspring age when comparing energy intake in growing female offspring, which represented increased energy consumption to support growth and development with age. All female offspring consumed more energy in later weeks of feeding (6<sup>th</sup> and 8<sup>th</sup> week of feeding), compared to earlier weeks of feeding (2<sup>nd</sup> and 4<sup>th</sup> week of feeding in offspring from dams fed HSF diet [ $p < 0.05$ ]; 2<sup>nd</sup> week of feeding in offspring from dams fed CON diet [ $p < 0.05$ ]), regardless of maternal diet. There were no differences in body weight in growing female offspring over the duration of the feeding period ( $p = \text{NS}$ ) (Figure 5).

#### 7.3.2. Fat pad masses of female offspring

There were significant main effects for offspring age when examining retroperitoneal and omental fat pad weights, both absolute weights and weights relative to body weight (g/kg). Female offspring at 3 months of age had higher absolute and relative levels of retroperitoneal ( $p < 0.001$ ) and omental ( $p < 0.001$ ) fat pad masses compared to



**Figure 5.** a) Energy consumption and b) body weight of female offspring. Values are expressed as mean  $\pm$  SEM.

at weaning. No comparisons were made for weanling parametrial fat pads as offspring have not reached reproductive maturity and negligible amounts of adipose tissue are stored in this depot (Table 7).

#### 7.3.3. Plasma hormones of female offspring

There was a significant interaction of maternal diet and age while comparing plasma MCP-1 concentrations ( $p = 0.026$ ) in female offspring (Table 7). At weaning, females from dams fed HSF diet had higher concentrations of MCP-1 than females from dams fed CON diet. Additionally at 3 months of age, females from dams fed HSF diet had higher concentrations of MCP-1 than at weaning. There were no differences in plasma leptin, IL-6,  $17\beta$ -estradiol or progesterone in female offspring at weaning or 3 months of age ( $p = \text{NS}$ ). Plasma concentrations of TNF- $\alpha$  were below the minimum level of detection for the assay, therefore no comparison was made (Table 7). However at weaning, a greater number of females from dams fed HSF diet ( $n = 3$ ) had detectable levels of TNF- $\alpha$  than females from dams fed CON diet ( $n = 0$ ).

#### 7.3.4. Body composition of female offspring

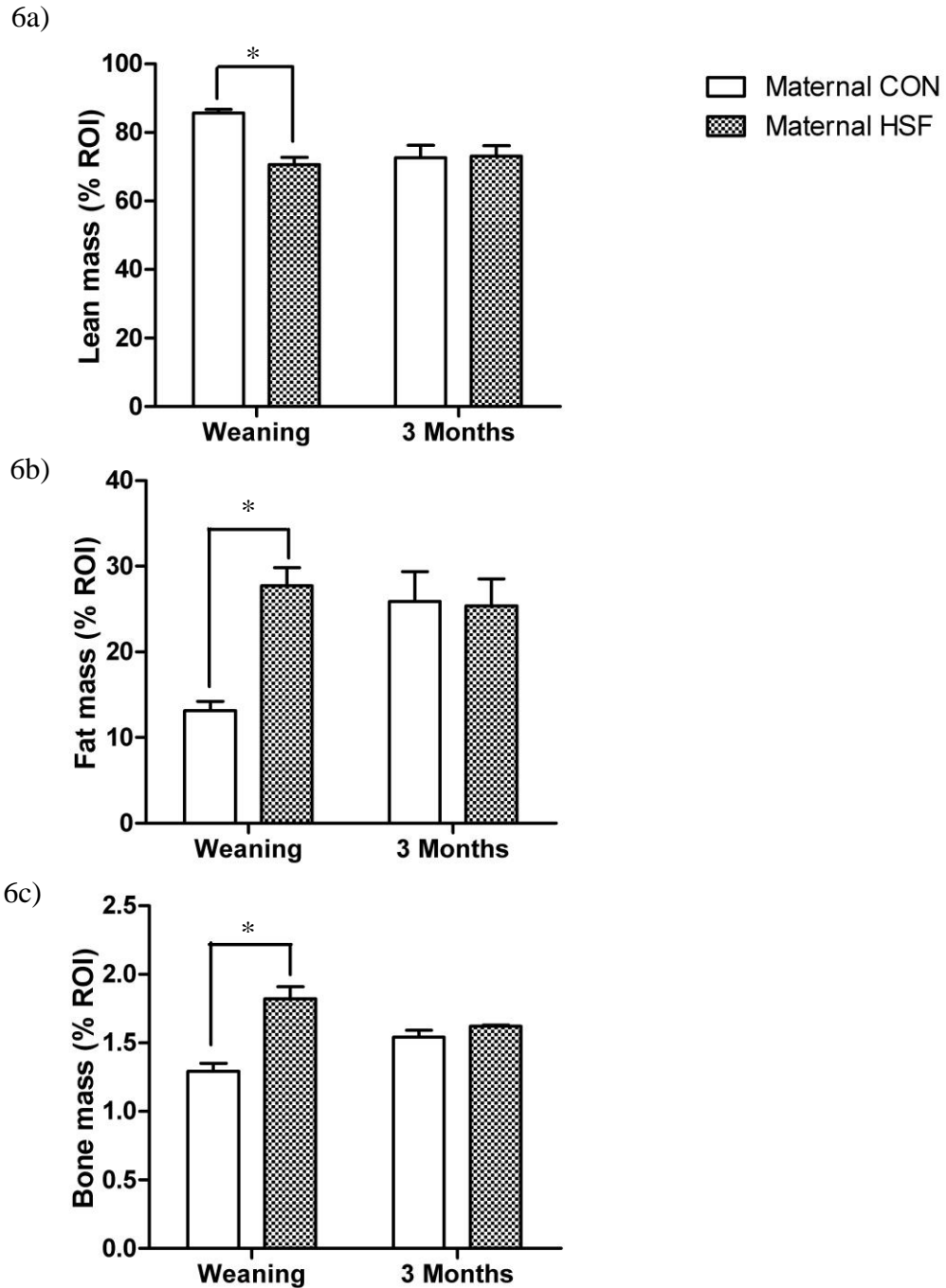
There were significant interactions of maternal diet and age for % lean mass ( $p = 0.013$ ), % fat mass ( $p = 0.014$ ), and % bone mass ( $p = 0.001$ ) in female offspring at weaning and 3 months of age (Figure 6). At weaning, females from dams fed HSF diet had lower % lean mass, with higher % fat and % bone mass than offspring from dams fed CON diet (Figure 6). Female offspring from dams fed CON diet at 3 months of age had lower % lean mass, and higher fat and % bone mass than at weaning. Moreover, females

**Table 7. Fat pad mass and plasma hormones in female offspring**

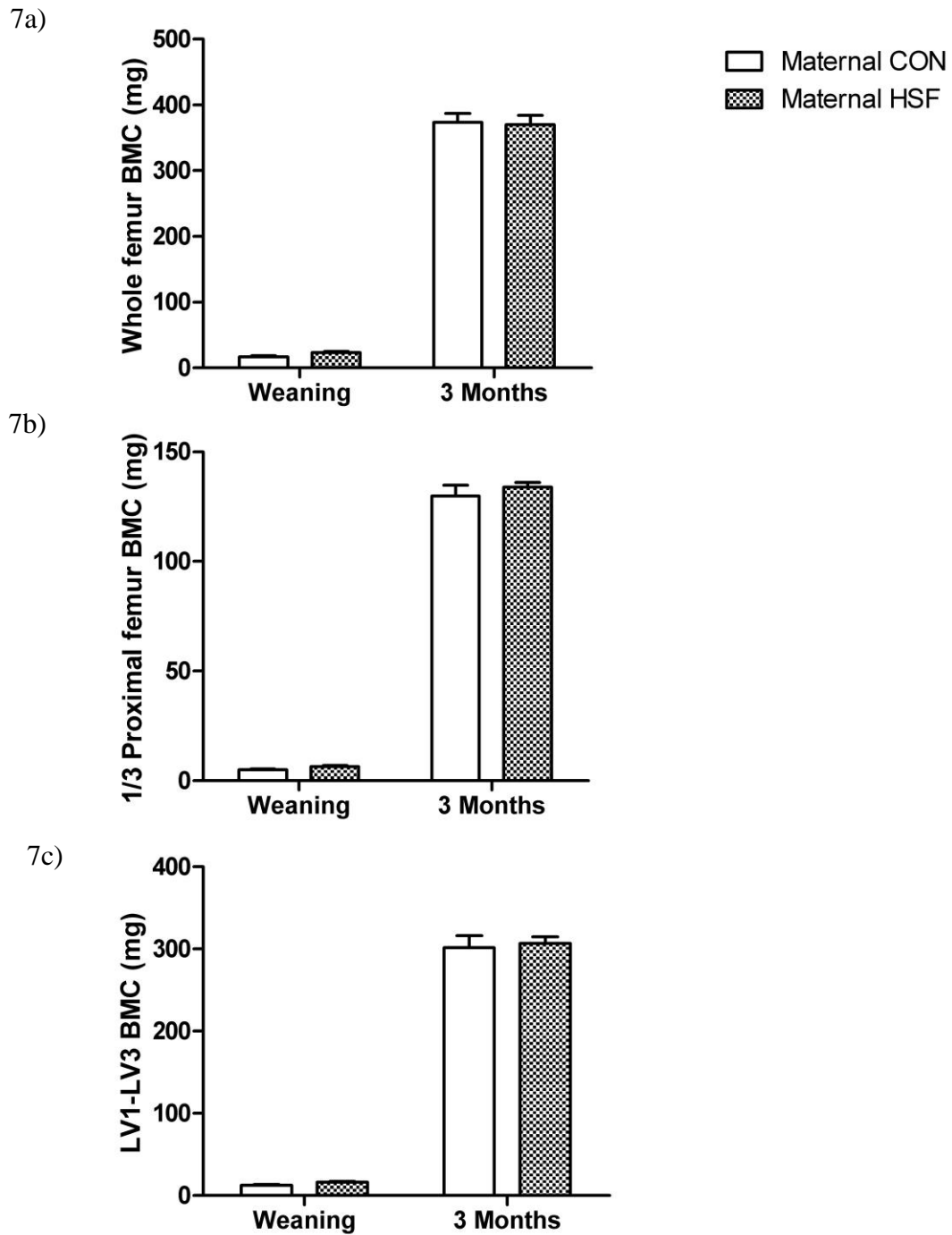
Outcomes	Weaning		3 Months		p Values	
	Maternal	Maternal	Maternal	Maternal	Diet	Age
	CON	HSF	CON	HSF	Diet	Age
<b>Fat pad mass</b>						
Retroperitoneal (g)	0.1 ± 0.02	0.2 ± 0.03	7.3 ± 0.5	8.7 ± 1.6	0.396	<0.001
(g/kg body weight)	2.3 ± 0.3	3.3 ± 0.5	23.6 ± 1.1	28.0 ± 4.9	0.310	<0.001
Omental (g)	0.3 ± 0.04	0.5 ± 0.05	6.8 ± 0.4	7.0 ± 0.5	0.518	<0.001
(g/kg body weight)	7.6 ± 0.9	9.7 ± 0.6	22.37 ± 1.4	22.8 ± 1.4	0.245	<0.001
Parametrial (g)	NM	NM	10.7 ± 0.6	9.5 ± 1.0	0.278	
(g/kg body weight)	NM	NM	34.9 ± 1.8	30.7 ± 2.8	0.233	
Total (g)	NM	NM	24.8 ± 1.2	25.2 ± 2.5	0.933	
(g/kg body weight)	NM	NM	80.9 ± 3.6	81.5 ± 7.0	0.933	
<b>Plasma Hormone</b>						
Leptin (pg/mL)	14343 ± 4102	12858 ± 6909	2763 ± 190	2678 ± 372	0.685	<0.001
TNF- $\alpha$ (pg/mL)	BD	BD	10.1 ± 1.6	10.2 ± 1.2	0.965	
IL-6 (pg/mL)	77 ± 16	159 ± 46	313 ± 56	305 ± 36	0.281	<0.001
MCP-1 (pg/mL)	282 ± 47	616 ± 149 <sup>a</sup>	293 ± 52	235 ± 36 <sup>b</sup>	0.113	0.036
17 $\beta$ -estradiol (ng/mL)	NM	NM	0.04 ± 0.01	0.05 ± 0.02	0.265	
Progesterone (ng/mL)	NM	NM	29.5 ± 5.9	19.4 ± 3.1	0.146	

Values are expressed as mean ± SEM (n = 9 per group); except for measures of retroperitoneal fat at weaning (n = 8 in offspring from dams fed CON diet), leptin (n = 6/group; 3 values per group exceeded highest standard). <sup>a</sup> Values are significantly different from maternal CON at weaning. <sup>b</sup> Values are significantly different from maternal HSF at weaning. NM, not measured due to small amounts of parametrial fat and blood volume in weanlings. BD, values below the level of detection, no comparison made.

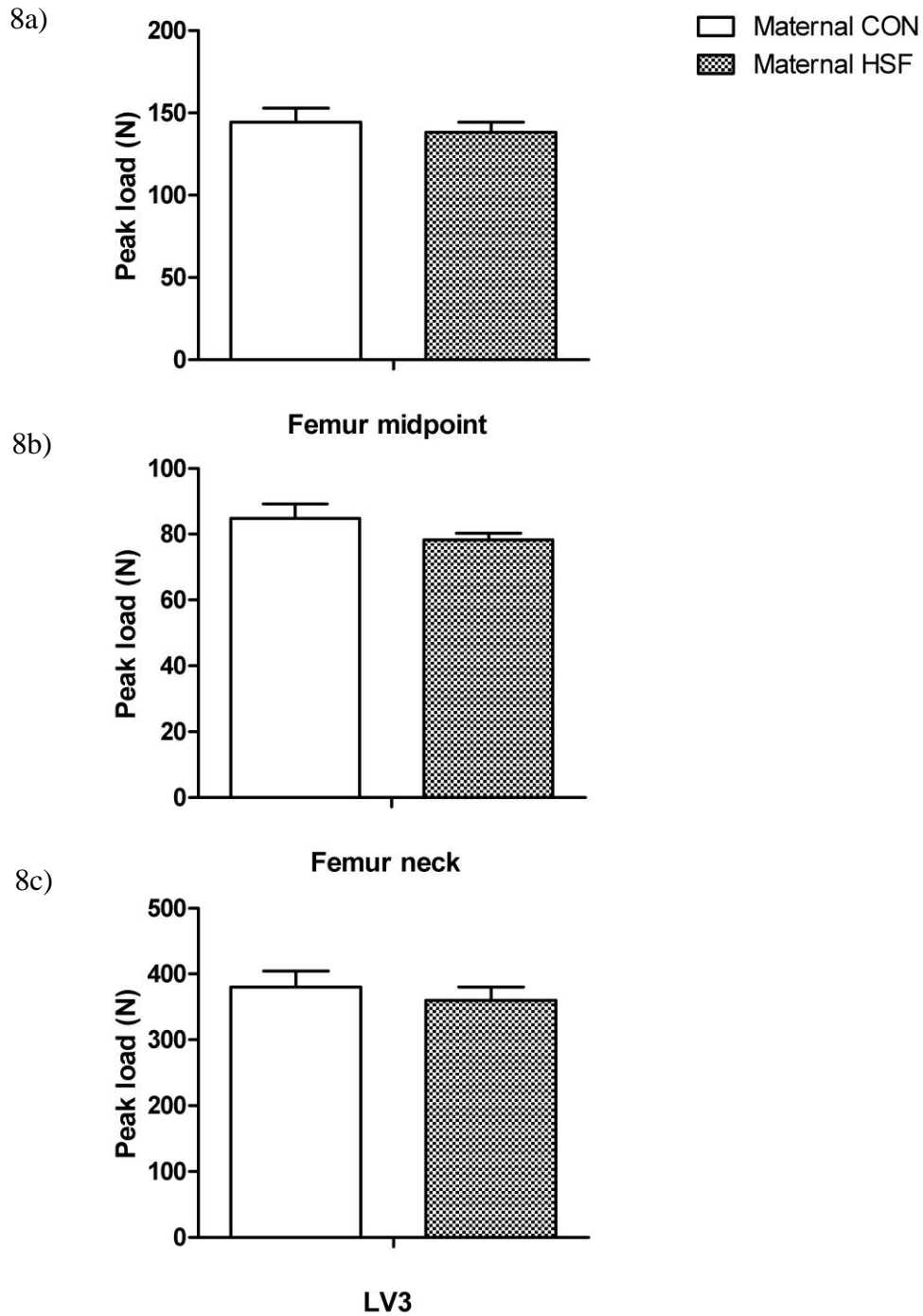




**Figure 6.** Body composition of female offspring, expressed as a percent of ROI: **a)** lean mass, **b)** fat mass, and **c)** bone mass. Values are expressed as mean  $\pm$  SEM. Weanling offspring,  $n = 7/\text{group}$ ; 3 month offspring,  $n = 9/\text{group}$ . \* Values are significantly different from maternal CON at weaning ( $p < 0.05$ ). CON: control diet; HSF: high saturated fat diet; ROI: region of interest.



**Figure 7.** Bone mineral content (mg) of female offspring: **a)** whole femur, **b)** 1/3 proximal femur, and **c)** LV1-LV3. Values are expressed as mean  $\pm$  SEM. Weanling offspring,  $n = 7/\text{group}$ ; 3 month offspring,  $n = 9/\text{group}$ . BMC: bone mineral content; CON: control diet; HSF: high saturated fat diet; LV1-LV3: lumbar vertebrae 1-3.



**Figure 8.** Peak load (N) of female offspring: **a)** femur midpoint, **b)** femur neck, **c)** LV3. Values are expressed as mean  $\pm$  SEM.  $n = 9/\text{group}$ , except LV3 peak load ( $n = 7/\text{group}$  as vertebral strength of 2 samples per group exceeded the load cell capacity of 475N). CON: control diet; HSF: high saturated fat diet; LV3: lumbar vertebrae 3.

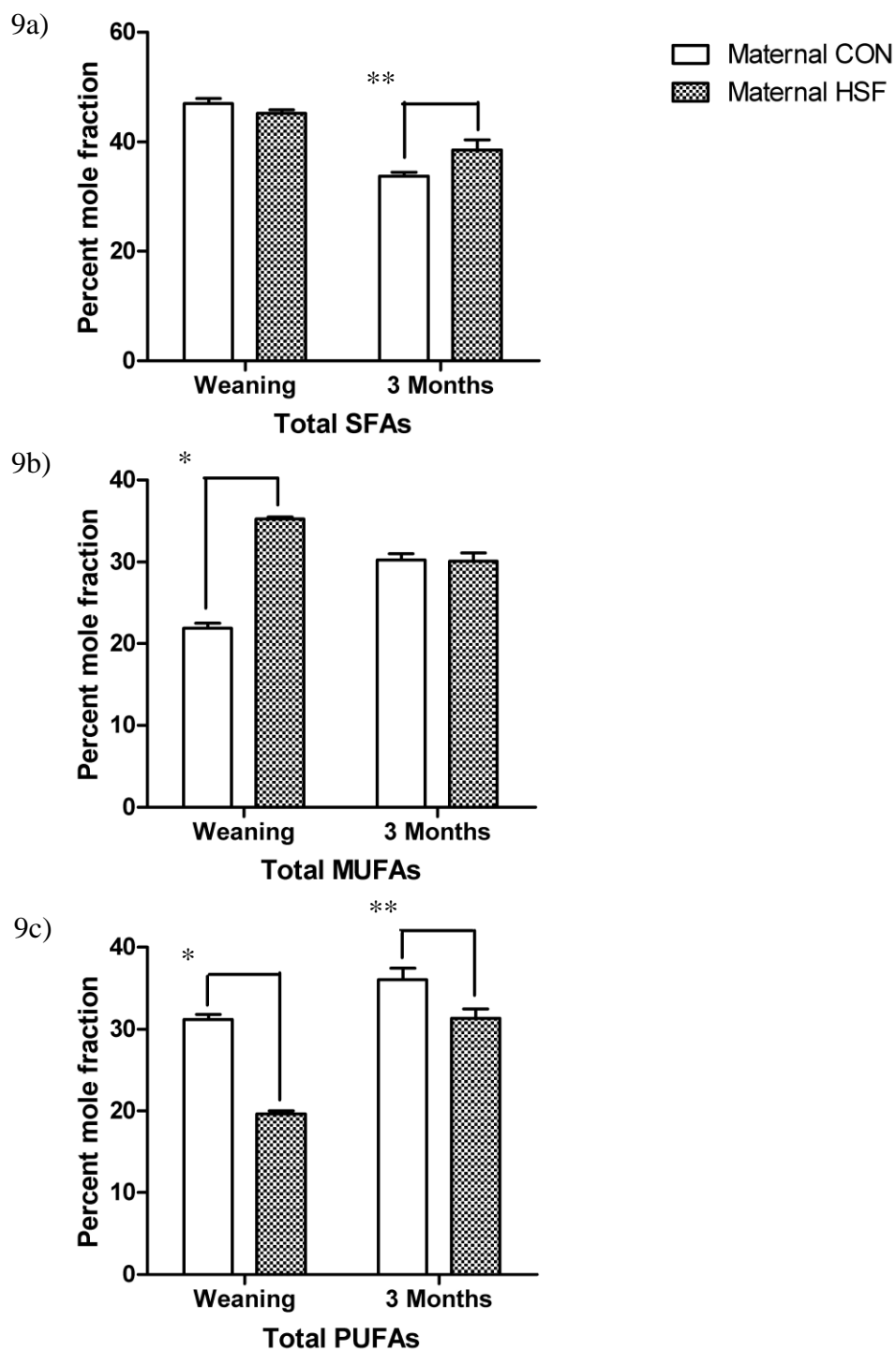
from dams fed HSF diet at 3 months of age had lower % bone mass than at weaning (Figure 5).

#### 7.3.5. Bone mineral and strength in femurs and lumbar vertebrae of female offspring

There was a significant interaction of maternal diet and age for whole femur BMD ( $p = 0.040$ ) (Figure 7). At weaning, females from dams fed HSF diet had greater BMD in the whole femur than females from dams fed CON diet. Females from dams fed CON diet at 3 months of age had greater whole femur BMD than at weaning. Similarly, females from dams fed HSF diet at 3 months of age had greater whole femur BMD than at weaning. There were main effects of age for BMC and BMD at all bone sites. Females at 3 months of age had higher BMC ( $p < 0.001$ ) (Figure 6) and BMD ( $p < 0.001$ ) (Figure 7) than females at weaning, regardless of maternal diet, due to growth. No differences were detected between groups in peak load at the femur midpoint ( $p = 0.114$ ), femur neck ( $p = 0.600$ ), or LV3 ( $p = 0.523$ ) in female offspring at 3 months of age (Figure 8).

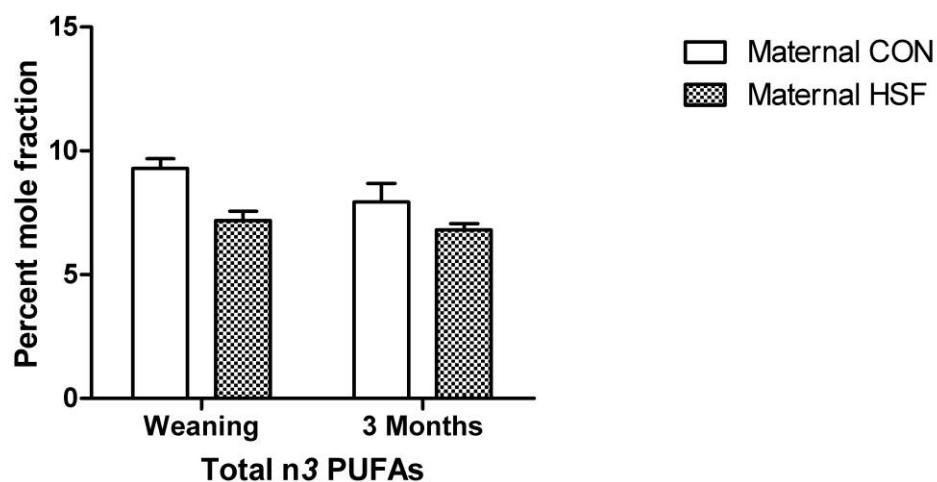
#### 7.3.6. Fatty acid composition of femurs from female offspring

There were significant interactions for maternal diet and age while examining percent mole fractions of total SFAs ( $p = 0.009$ ), MUFAs ( $p < 0.001$ ), PUFAs ( $p = 0.002$ ) (Figure 9) and  $n6$  PUFAs ( $p < 0.001$ ) (Figure 10) in femurs of female offspring. At weaning, females from dams fed HSF diet had higher total MUFAs, with lower total PUFAs and  $n6$  PUFAs. Females from dams fed CON diet at 3 months of age had lower total SFAs, and higher total MUFAs, total PUFAs, and  $n6$  PUFAs than at weaning. Similarly, females from dams fed HSF diet at 3 months of age had lower total SFAs and

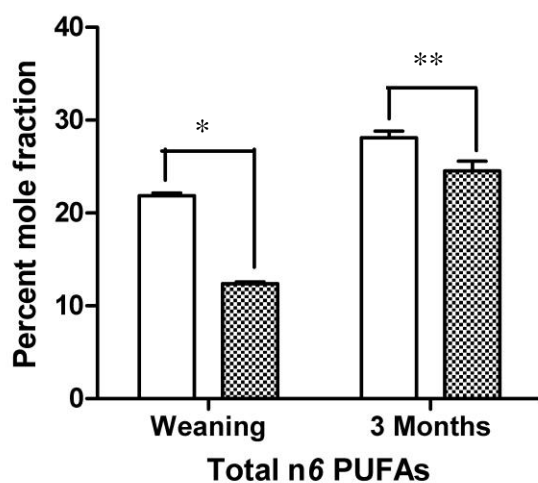


**Figure 9.** Percent mole fractions of fatty acids in femurs from female offspring: **a)** total SFAs, **b)** total MUFAs, and **c)** total PUFAs. Values are expressed as mean  $\pm$  SEM.  $n = 8-9/\text{group}$ , \* Values are significantly different from maternal CON at weaning ( $p < 0.05$ ). \*\* Values are significantly different from maternal CON at 3 months ( $p < 0.05$ ). CON: control diet; HSF: high saturated fat diet; SFAs: saturates; MUFAs: monounsaturates; PUFAs, polyunsaturates.

10a)



10b)



**Figure 10.** Percent mole fractions of PUFAs in femurs from female offspring: **a)** total *n*3, and **b)** total *n*6. Values are expressed as mean  $\pm$  SEM. *n* = 8-9/group. \* Values are significantly different from maternal CON at weaning ( $p < 0.05$ ). \*\* Values are significantly different from maternal CON at 3 months ( $p < 0.05$ ). CON: control diet; HSF: high saturated fat diet; PUFAs, polyunsaturates.

total MUFAs, with higher total and *n*6 PUFAs than at weaning. At 3 months of age, females from dams fed HSF diet had higher total SFAs, with lower total (Figure 9) and *n*6 PUFAs (Figure 10).

## **Chapter 8.0: Discussion**

The results of this study demonstrate that female Wistar rat offspring body composition and bone health are not negatively impacted by a single generation of maternal consumption of a HSF diet. Differences in body composition and bone health at weaning were not sustained at 3 months of age; therefore the elevated level of fat in the maternal dietary intervention did not contribute to long-lasting programming effects in female offspring. Alterations in femur fatty acid composition due to maternal consumption of a HSF diet persisted to 3 months of age. Further study is required to determine if and when fatty acid composition of the femur reflects the diet consumed. Despite the persistent changes in femur fatty acid composition, there were no differences in femoral and lumbar vertebrae peak load in female from mothers fed a HSF diet at 3 months of age suggesting that fatty acid composition of bone may not be directly associated with bone strength.

### **8.1. Female offspring**

#### **8.1.1. Food intake, body weight, and fat pad masses**

Differences in food intake in female offspring were apparent at 4, 6 and 8 weeks of feeding post-weaning, regardless of maternal diet. In both groups of offspring, post-weaning energy consumption was higher at weeks 4, 6 and 8 of feeding compared to week 2. This may be an effect of growth and development, where offspring need to consume more food to support rapid growth during this time. This finding is also in accordance with the female offspring body weight. Although weanling females from dams fed the HSF diet were heavier than those from dams fed CON diet, these

differences were diminished over the feeding period. Similarly, there were significant differences in fat pad mass, in absolute values and adjusted for body weight, between all offspring at 3 months of age compared to weaning. However this was only an effect of offspring age and growth, and not necessarily maternal diet. It may be that weanling females from mothers fed the high fat diet were heavier with greater fat mass than females from mothers fed CON diet due to the greater availability of nutrients from mother's milk during suckling. It is speculated that the milk from mothers fed the HSF diet would have contained more total energy compared to milk from mothers fed control diet, with a higher level of fat, protein, and other important nutrients [17, 18]. Therefore, exposure to mother's milk during suckling in females from mothers fed the HSF diet may have contributed to greater weight gain and level of fat mass.

#### 8.1.2. Body composition

Body composition of female offspring was also impacted by the maternal HSF diet at weaning. Weanling females from dams fed a high fat diet had lower % lean mass, and higher % fat and % bone mass than females from dams fed CON diet. The findings for % lean mass and % fat mass are consistent with current literature [17, 150]. This is the first study to report % bone mass in weanling female offspring as a result of a maternal HSF diet. Higher % bone mass in weanling females from dams fed HSF diet may be due to greater nutrient availability provided in mother's milk during suckling, as offspring are consuming a diet similar in composition to the diet of the mother. This has been demonstrated in previous studies that have shown the composition of mother's milk reflects the diet consumed [17, 18], and therefore may contribute to greater availability of



nutrients involved in bone mineral acquisition such as calcium and vitamin D. Despite these findings, observed differences in body composition between groups at weaning were not sustained at 3 months of age, indicating the maternal high fat diet did not contribute to long-lasting changes in female offspring body composition. Differences observed at weaning may be attributed to the greater availability of nutrients provided in the dam's milk (i.e. increased fat and protein [17, 18]). Any effects observed at 3 months of age may have been attributed to growth and development. Females from dams fed CON diet at 3 months of age had lower % lean mass, with higher % fat and % bone mass than at weaning. Additionally, 3 month old females from dams fed HSF diet had lower % bone mass than at weaning. These apparent findings cannot be attributed to maternal diet, but rather offspring growth and development.

#### 8.1.3. Plasma hormone concentrations

Maternal high fat diet consumption influenced plasma levels of MCP-1 at weaning, where females from dams fed HSF diet had higher levels than females from dams fed CON diet. This elevation in MCP-1 however, did not persist at 3 months of age in females from dams fed HSF diet, as levels were significantly lower than MCP-1 levels in offspring at weaning. Therefore there was no programming effect on MCP-1 levels due to maternal consumption of a HSF diet. Differences in weanling MCP-1 plasma levels may be attributed to the higher fat content in mother's milk during suckling. These results are also in accordance with body composition at weaning, where females from dams fed HSF diet had greater % fat mass, as levels of MCP-1 are positively correlated with overall fat mass [192]. Furthermore, the finding that MCP-1 levels are lower at 3 months

of age than at weaning in females from dams fed HSF diet is also in accordance with adiposity, where elevations in % fat mass at weaning did not persist to early adulthood.

#### 8.1.4. Bone mineral, strength, and fatty acid composition

Femoral bone mineral and fatty acid profile in females at weaning and 3 months were also impacted by maternal HSF diet. The finding that whole femur BMD in weanlings from dams fed HSF diet was greater than females from dams fed CON may also be attributed to greater nutrient availability from mother's milk during suckling, allowing for greater bone mineral acquisition. Interestingly, this effect was only found in BMD of the whole femur. However there was no programming effect of maternal HSF diet on offspring whole femur BMD as these differences were not sustained at 3 months of age. Fatty acid composition of femurs at weaning reflected the fatty acid composition of the diet consumed, which is in agreement with previous studies in older rats where dietary fat has been manipulated [63, 190]. Furthermore, it is established that different skeletal sites, such as the femur and lumbar vertebrae, respond similarly to variations in dietary fatty acid composition [190, 193, 194]. Interestingly, changes in femur fatty acid composition at weaning were sustained at 3 months of age, with additional elevations in SFAs as well. Regardless of these findings, there were no functional differences in femoral bone strength, suggesting that increased total SFA content may not contribute to increased fracture risk at early adulthood. It may also be that changes in bone fatty acid composition may not necessarily be detrimental to bone health, and may not lead to changes in bone strength. However this is speculative and further research in this area is needed.

Typically, bone health is a measure of bone quantity (mineral), quality (microarchitecture) and strength. However for the purpose of this study, we did not measure bone quality. We measured bone strength, a predictor of fracture, after measuring bone mineral, to understand whether there were functional differences that rendered the bones weaker and/or stronger due to maternal consumption of a HSF diet. Since both bone quantity and quality are predictors of bone strength, we were able to appropriately determine that there was no evidence of programming, as there were no differences in quantity or strength.

## **8.2. Dams**

### **8.2.1. Pregnancy outcomes and litter characteristics**

Many of the outcomes measured in female offspring were also measured in dams, to appropriately determine the extent to which dam body weight and overall bone health impacted offspring health. It is well established that a maternal HSF diet can impact fertility and has been shown to result in unsuccessful pregnancies in rodents [195]. In this study, only 3 dams fed the HSF diet and 2 dams fed CON diet did not get pregnant, therefore a maternal high fat diet did not negatively impact fertility in dams over the studied term. This finding was also shown in other studies using rodents that implemented lard as the primary fat source in the HSF diet [196]. A maternal HSF diet did not impact male:female ratio and litter size, which has been shown previously [17, 196, 197]. Significant differences in litter weight were apparent at PND12, which was likely the result of greater nutrient delivery from mother's milk. Differences in litter

weight were sustained at weaning [44, 197], which may have been due to greater nutrient delivery from mother's milk and possible consumption of mother's diet.

#### 8.2.2. Food intake, body weight, fat pad masses, and plasma hormone concentrations

Dams fed the HSF diet had greater energy consumption in 8 of 10 weeks of feeding compared to dams fed CON diet. Dams fed the HSF diet may have compensated food intake for the increased energy content of the diet, as they consumed less food (grams per rat per day) than dams fed CON for 6 of 10 weeks. This may have contributed to the lack of obesity found in dams as they were able to self-regulate food intake.

In addition to a lack of obesity, there were no differences in body weight for 10 weeks of feeding before breeding, however dams that consumed HSF diet seemed to gain more weight during gestation and lactation as body weight at necropsy was significantly higher, as shown previously [150], but were not obese. Recent studies in rodents have speculated that maternal obesity rather than diet is the key factor for inducing programming in offspring [198]. With this in mind, the lack of maternal obesity in this study may have contributed to the absence of programming in female offspring body composition and bone health. The lack of significance in fat pad masses (absolute and adjusted for body weight) and plasma hormones are in accordance with body weight and food intake data.

#### 8.2.3. Bone mineral, strength, and fatty acid composition

As hypothesized, the fatty acid composition of femurs reflected that of the diet consumed with greater SFA content. Although previous studies have shown that a high

fat diet can lower BMC, BMD [70], structure [43] and even strength in rodents [66], there were no differences in bone mineral (BMC, BMD) and strength in femurs and lumbar vertebrae. Therefore higher SFA content in femurs does not contribute to functional differences in bone strength. Whether higher SFA content can alter bone structure in dams and offspring remains to be determined.

## **Chapter 9.0: Conclusions**

A maternal HSF diet modified with higher levels of protein, vitamins, and minerals, does not elicit long-term changes in female offspring body composition (increased fat mass, lower lean and bone mass) or bone health (lower bone mineral and peak load) at 3 months of age. Although female offspring at weaning from mothers fed the HSF diet had higher fat mass and bone mass, with lower lean mass compared to female offspring from mothers fed CON diet, this did not persist to 3 months of age. Furthermore, fat pad masses were not different in offspring at weaning and 3 months of age. There were no differences in BMC in the femur or lumbar vertebrae, however females from mothers fed the HSF diet had higher femoral BMD at weaning, but not 3 months of age. Femur fatty acid composition of weanling female offspring from mothers fed a HSF diet reflected maternal diet (higher total MUFAs, lower total and *n*6 PUFAs) from exposure during suckling. However these alterations in femur fatty acid composition (lower total and *n*6 PUFAs) were persistent at 3 months of age, with additional increases in higher SFA content. We speculate this may not be a true effect of programming; rather females are still growing at 3 months of age and may use these fat stores to support further bone growth. Even considering these consistent alterations in

femur fatty acid composition, there were no differences in femoral strength, or vertebral strength. Therefore, although exposure to the HSF diet during suckling did promote changes in body composition and bone health at weaning, it appears there was no programming, as these effects were not observed at 3 months of age. This is an indication that a healthy post-weaning diet may prevent prolonged effects of maternal HSF diet consumption on female offspring body composition and bone health.

### **9.1. Strengths**

There are many unique aspects of this research that allowed us to determine whether maternal HSF diet consumption was a major contributor to changes in female offspring body composition and bone health. This has been the only research to examine female offspring at two different life stages, early life (weaning) and young adulthood (3 months of age). Although one study has examined female offspring at two time points, 3.5 and 6.5 months of age [152], this does not represent two unique life stages, as offspring are in adulthood at both time points. We believe it is important to study offspring at early life and early adulthood, as these two life stages represent two different rates of growth, fast (early life) and slow (early adulthood).

A major strength of this study was that the level of protein, vitamins, and minerals were increased in the HSF diet. To reiterate, this was done to compensate for the increased energy in the diet provided by the greater level of fat. This compensation, or nutrient adjustment, kept the relative energy contribution of these important nutrients similar to that of a healthy diet. Overall, this ensured that any observed effect on female offspring body composition and bone health was truly attributed to maternal HSF diet

consumption, and not due to lower intake of these nutrients on an energy basis. This is especially important as the consumption of protein, vitamins (e.g. vitamin D), and minerals (e.g. calcium) are essential for optimal bone health [69, 153, 174, 177, 199]. Although one study did also increase the level of protein, vitamins, and minerals in the high fat diet [58], there was no dietary control group in which the results could be appropriately attributed to maternal high fat diet consumption (i.e. female offspring from mothers fed a high fat diet were *also* fed the high fat diet). In addition to the adjustment of important nutrients, we had dietary interventions that allowed us to tease out the true effect of maternal HSF diet consumption (i.e. female offspring from mothers fed a HSF diet were fed a healthy *control* diet).

This research is also novel in that it examined the impact of maternal HSF diet consumption on female offspring body composition *and* bone health. Most studies have investigated either body composition [17, 44, 149, 151] *or* bone health [58] in female offspring. Investigating both female offspring body composition and bone health provides a greater understanding of the health status of female offspring as a whole, rather than specific to certain tissue. Moreover, this research is the first to investigate fatty acid composition in female offspring bones as a result of maternal HSF diet. With this novel information, we were able to determine that although there may have been changes in fatty acid composition that were persistent at 3 months of age, this did not translate into functional differences in strength.

## **9.2. Limitations**

The current study had a few limitations that should be taken into consideration. Firstly, although we observed changes in female offspring body composition at weaning, we did not examine the composition of mother's milk. Knowing the nutrient content in terms of macronutrients, vitamins, and minerals may have helped to understand the underlying changes in female offspring body composition and BMD at weaning. Secondly, markers of bone formation and bone resorption were not examined in addition to measurements of bone mineral and strength. These markers could help determine whether the increased femoral BMD in weanling female offspring was due to the up-regulation of bone formation, or a result of nutrient delivery.

### **9.3. Future Implications**

Based on the conclusions of this study, it seems that a healthy diet post-weaning may prevent long-lasting changes in body composition and bone health in female offspring, despite being born to mothers fed a HSF diet. This research will provide further insight to the impact of maternal HSF diet consumption on female offspring at weaning and adulthood, which is important considering the numerous discrepancies in the literature. Long-term, the findings from this study may help to develop nutritional strategies and dietary recommendations for the general public. This research may also help parents promote healthy eating and overall optimal health in their children.

### **9.4. Future Research**

Future research in the area of offspring health due to maternal HSF diet consumption should continue, considering the high incidence of obesity and osteoporosis



in Canada [1, 200]. To have a greater understanding of female offspring body composition and bone health due to maternal diet, future investigations should consider the following:

*i. Investigation of bone microarchitecture using micro-computed tomography.* Bone strength measurements in weanling offspring were difficult to obtain in this study, as femurs were still immature. Future investigation of bone architecture would provide additional information on the impact of maternal HSF diet consumption on bone health, in addition to measurements of bone mineral. Further research is required to determine whether bone microarchitecture is altered as a result of maternal HSF diet consumption. Although some studies have investigated bone microarchitecture in female offspring at multiple sites, these studies did not have: 1) experimental groups designed to ensure that observed effects were truly attributed to maternal diet (i.e. offspring from mothers fed a high fat diet were also fed a high fat diet post-weaning) [58]; and 2) increased the level of protein, vitamins and minerals to account for the increased energy provided by the elevated fat in the diet [152]. Determination of bone microarchitecture at multiple sites, with study designs that include both factors listed above, will allow for a greater understanding of the impact of maternal HSF diet consumption on female offspring bone structure.

*ii. Mechanisms underlying changes in bone growth.* Future studies in this area of research could investigate specific changes in bone development by measuring transcription factors governing bone growth and differentiation (i.e. Runx2, osterix). This would help determine whether increases in bone mineral, as determined in this study (in weanlings from mothers fed a high fat diet), are attributed to greater nutrient availability from

mother's milk, or due to upregulation of these transcription factors that promote bone formation.

*iii. Storage location of altered bone fatty acids.* Considering that this study showed persistent alterations in bone fatty acid composition in offspring at early adulthood without changes in bone strength, it is important to understand the underlying mechanisms. Although there is evidence of changes in total bone fatty acid composition, it is unclear where these lipids are stored and where these alterations have occurred. No study has yet to investigate the location of storage of these fatty acids in bone. Perhaps details into lipid storage in bone will help determine the underlying reason why changes in bone fatty acid composition do not translate into differences in bone strength.

*iv. Paternal consumption of a HSF diet.* It may also be important to determine whether there is a paternal influence, in addition to maternal, on programming of female offspring body composition and bone health. This is important considering the increased prevalence of obesity in Canadian men [200]. Furthermore, recent evidence shows that paternal consumption of a high fat diet can predispose offspring to become obese in adulthood, which may have been facilitated by epigenetic changes [201]. Therefore, it may be important to determine the direct impact of paternal HSF diet consumption on female offspring body composition and bone health, and whether these effects are further exacerbated when mothers also consume a HSF diet.

## **Chapter 10.0: Bibliography**

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